UNIVERSITA' DEGLI STUDI DI PISA

SCUOLA DI DOTTORATO DI RICERCA IN BIOLOGIA

CICLO XXI - 2006

PhD Thesis:

Detoxification mechanisms in different taxa of autotrophic protists under heavy metal stress

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ABSTRACT

For the comprehension of the processes of transport, transformation and accumulation of heavy metals in the marine environment an important contribute lies on the understanding of the diverse strategies developed during evolution by aquatic and terrestrial organisms in order to maintain an equilibrated relation with heavy metal ions present and available in the surrounding medium. The aim of the present study was to investigate the main mechanisms of detoxification acting in autotrophic protists to cope with heavy metal stress. Since a general response to metal stress in autotrophic organisms is represented by the synthesis of metal-binding peptides, named phytochelatins (PC), this study was focused on the induction of these peptides in different taxa of marine phytoplanktonic protists. Initially the induction of PC was investigated in cells of the marine diatom *Phaeodactylum tricornutum* exposed to environmentally relevant levels of dissolved Cd, Cu, Pb and Zn by performing short-term incubations both in EDTA-buffered artificial seawater and in natural seawater samples. Results showed that PC behave as a biomarker of exposure to the bioavailable metal fraction. Successively, experiments were carried out to examine the PC response in 5 taxa of autotrophic protists exposed to Cd, Cu and Pb. Results showed that the PC response depends on the particular species, the chemical form of the metal, the time of exposure and the metal concentration in the external medium. Successively, the research was devoted to develop new bioassays involving the presence of PC in phytoplankton as biomarkers of metal bioavailability in marine waters and sediments. The results obtained strongly support the feasibility of using this biochemical response in the assessment of toxicity of environmental systems. Besides the ability to synthesize metal binding peptides, the capability to produce Dissolved Gaseous Mercury (DGM) as another defence mechanism was investigated in *Thalassiosira weissflogii* exposed to potentially toxic concentration of mercury. Mercury exposure experiments needed a separate study given the chemical particularities of this metal in comparison to the other heavy metals and the peculiarity of the experimental methodology necessary for mercury determination. The results showed that the diatom responded to mercury exposure by synthesizing PC, besides to increase the intracellular pool of glutathione and γ-Glu-Cys. The time course of the non protein thiols pool and Hg intracellular concentration showed that PC, glutathione and γ-Glu-Cys represent a rapid cellular response to mercury exposure; however, at longer incubation times, their role in Hg detoxification seems to lose importance. At lower Hg concentration, at which the PC synthesis doesn't seem to be involved, the occurrence of a process of reduction of the DGM production was investigated in the same diatom. The significant correlation between the cellular density in solution and the production of DGM, both in light and dark conditions, clearly showed that *T. weissflogii* is capable to directly produce DGM. This finding has been confirmed by the absence of DGM production in culture media containing formaldehyde-killed cells of *T. weissflogii*. This approach is part of a wider study regarding the contribution of the eukaryotic and prokaryotic microorganisms to the production of DGM in aquatic systems.

Keywords: heavy metals; autotrophic protists; detoxification mechanisms; phytochelatins; dissolved gaseous mercury

CHAPTER 1

INTRODUCTION

1.1. Heavy metals in marine environment

Heavy metal pollution due to human activities represents a considerable concern for the modern world. Although human activities have always impacted on coastal areas, it is only within the last two centuries that the effects of industrialization, intensive agriculture and coastal engineering have seriously begun to threaten marine life (His et al., 1999). Forstner and Wittmann (1979) stated that compared with land systems, the relatively small biomass in aquatic environments generally occurs at a greater variety of trophic levels. This correlates to the particular sensitivity of aquatic systems with regard to pollution influences. Many substances pollute the marine environment, but non-biodegradable compounds are the most dangerous due to their innate ability to constantly remain with the ecosystem (Hernandez-Hernandez et al., 1990). In the last thirty years or so, heavy metals have become an increasingly common contaminant of sea and freshwater. Lakes and their sediments have long been recognized as common sinks for metals. Rivers, similarly, are capable of transporting large quantities of metals and constitute inputs for the marine environment. So the highest contamination levels of marine environment can be found in coastal and estuarine waters affected by the presence of anthropogenic activities and riverine inputs. Ober et al. (1987) affirmed that pollution of the marine ecosystem by heavy metals is a worldwide problem and the main sources of metal pollution are domestic/industrial sewage, industrial effluents, oil and chemical spills, combustion emissions, mining operations, metallurgical activities and non-hazardous landfill sites. According to Hernandez-Hernandez et al. (1990) the presence of metals in the marine environment is partly due to natural processes such as volcanic activity and erosion, but mostly results from industrial processes, with metals mainly entering the sea

suspended in industrial wastes and in solid particles carried by winds, and eventually deposited in the sea (Jackson et al., 2005). Metals are usually present at low or very low concentrations in the oceans (Morel and Price, 2003). The concentrations of total dissolved cadmium, lead, copper and zinc measured in surface seawaters are in the range 0.01 - 0.2 nM, 0.05 - 0.4 nM, 1 - 5 nM and 1 - 40 nM, respectively (Millero and Sohn, 1992), but they can reach values 50-100 times higher in polluted and industrialized coastal areas. Sediments represent the major sink for contaminants in aquatic systems and they are the main location of the heavy elements in the hydrosphere; subsequently sediment re-suspension can act as source of contaminants for the overlying water column. Sediment disturbance, by way of natural factors, or human activities, can cause changes in the chemical properties of sediment, bringing to the mobilization of contaminants that could pose a threat to living organisms (Forstner and Salomons, 1991). Hence, the analysis of sediments yields useful information on the metal burden of natural waters.

Some metal ions are essential trace elements, but, essential or not, most heavy metals are toxic at higher concentrations. They appear to be dangerous pollutants since they have not only a short-term toxic effect on human and aquatic organisms, but also a dangerous mutagenous, embryo-toxic and gonadotoxic long-term effect. The hazard of toxic metals is due to the fact that the biologically available forms of heavy metals in the environment can be transformed and accumulated by various organisms. The fate and distribution of the heavy metals in the aqueous environment are determined by a large number of reactions occurring with dissolved inorganic and organic ligands as well as with natural heterogeneous compounds such as mineral surfaces and biological particles (Scarano and Morelli, 1999). The ability of some aquatic organisms to take up and store heavy metals and other chemical substances occurring in the environment is widely recognized. Thus heavy metals enter nutrition chains and potentially endanger living beings, becoming concentrated in fish and

other edible organisms (known as bioaccumulation), particularly in near-shore areas (His et al., 1999). Overall, toxic effect of heavy metals occur as the result of a complex balance between the chemical speciation of the metal ion and the biology of the organism. There is now considerable evidence to suggest that the distribution and speciation of trace metals in the upper water column play an important role in the species composition and physiology of phytoplankton assemblages (Sunda 1994). The influence of metals and aquatic microorganisms is reciprocal: biological production in the oceans can strongly influence the oceanic chemistry of trace metals and, in turn, bioactive metals may affect the oceanic primary productivity.

Autotrophic microorganisms affect trace metal chemistry in natural and oceanic waters not only by surface reactions, but also by metal uptake and by production of extracellular organic matter capable to bind metal ions (Zutic et al., 1981; Seritti et al., 1986). Both the direct extracellular exudation products and the secondary products after biochemical modification have been demonstrated to have metal complexing properties (Imber et al., 1985; Zhou and Wangersky, 1985, 1989; Seritti et al., 1986). Thus, in natural waters, the production of the biogenic organic matter, by influencing the chemical forms of metal ions in solution, could affect the adsorption process on the cell surface (Scarano and Morelli, 1999).

1.2 Biogeochemical cycle of mercury

One significant aspect of the global biogeochemical cycling of mercury, that is different to other metals, is the volatility of this metal.

The natural sources of mercury emissions are weathering of rocks, windblown dust, volcanic activity, geysers, thermal fluids, degassing of the earth's mantle, emanations from the oceans, transpiration and decay of vegetation and forest fires. The main transport pathway for

mercury is through the atmosphere and for this reason mercury represents a powerful and dangerous pollutant that has become widespread throughout the world-wide environment.

The volatilization of mercury may occur at any stage in the transport process. Volatile species are produced by chemical or biochemical reduction of Hg^{2+} to Hg^0 and by the biomethylation of mercury to give dimethylmercury. Elemental mercury is a highly volatile chemical species and can be spread into the atmosphere even very far from the emission source (Schroeder et al., 1989).

The flux of mercury from the sea surface depends on the formation in the water column of volatile dissolved forms of mercury (90% elemental mercury), named Dissolved Gaseous Mercury (DGM), which pass from the water into the atmosphere due to their low water solubility (60µg/L at 25 °C) and high volatility (Henry coefficient < 0.3).

Mercury evasion from aquatic surfaces plays an important role in the complex biogeochemical cycle of this metal (Fig.1.1).

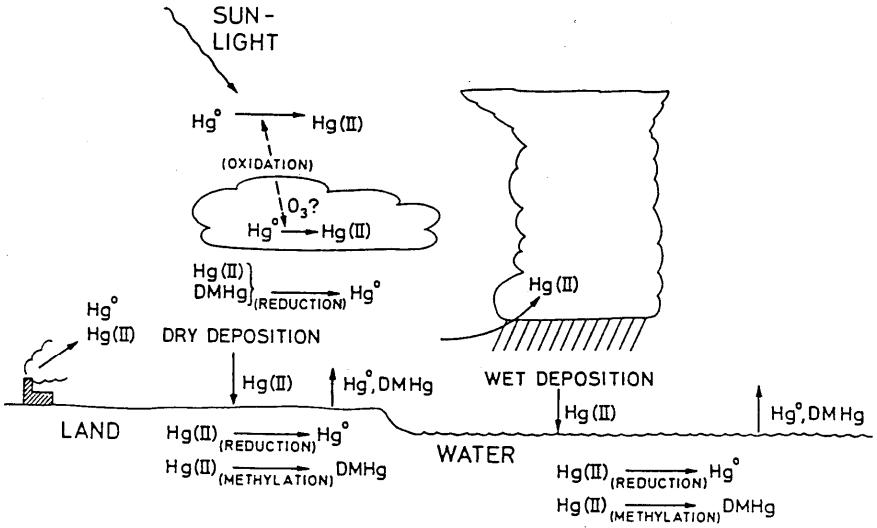

Fig. 1.1. Transports and transformations in the biogeochemical cycle of mercury (Lindqvist, 1994).

Even if the regional and global atmospheric budgets need to be reassessed (Gustin et al., 2000), the oceanic evasion of the DGM into the atmosphere is a phenomenon comparable to terrestrial emissions (Lindberg et al., 1995; Gardfeldt et al., 2003). In particular, the mercury emission from the entire Mediterranean Sea has been estimated to be about 60 tons per year (Ferrara et al., 2000a); therefore the aquatic evasion represents the main natural source of this metal into the atmosphere in the Mediterranean area, higher than the emission from volcanoes (0.6-1.3 t/y; Ferrara et al., 2000b).

The Mediterranean basin is characterised by the presence of a noteworthy geochemical anomaly with large cinnabar deposits (Fig. 1.2) (Bernhard and Renzoni, 1977; Buffoni et al., 1982; Ferrara et al., 1997), by intense solar radiation and high environmental temperatures for many months of the year. These characteristics induce the formation of elemental mercury and its emission from soil and surface water through biological, photo-chemical and photo-physical processes (Ferrara et al., 1997).

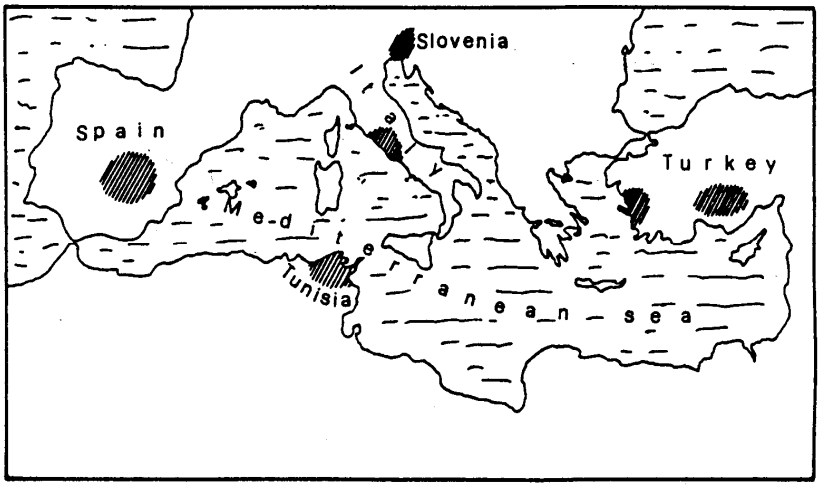

Fig. 1.2. The belt of cinnabar deposits in the Mediterranean basin.

1.2.1. Abiotic and biotic processes of Dissolved Gaseous Mercury production

The capability to produce Dissolved Gaseous Mercury (DGM), holds a noteworthy ecological importance in the context of the biogeochemical cycle of mercury. In aquatic systems the DGM formation and its volatilization to the atmosphere is a phenomenon comparable to terrestrial emissions (Lindberg et al., 1995; Gardfeldt et al., 2003); moreover, through DGM production, mercury is removed from the water column and is no longer available for methylation processes and subsequent bioaccumulation throughout food chains (Fitzgerald, 1993).

The dynamic of DGM formation results from several oxidation and reduction processes occurring simultaneously.

Literature records contrasting papers concerning the mechanisms of DGM formation: for a long period, authors have tried to establish if the production of elemental mercury can be defined "abiotic" or "biotic". There is evidence for a photochemically enhanced abiotic production of DGM (Nriagu, 1994; Xiao et al., 1994), proved by the existence of a daily and seasonal behaviour of the DGM concentration in lakes, estuaries, riverine waters (Amyot et al., 1994, 1997; Krabbenhoft et al., 1998; Amyot et al., 2000; Zhang and Lindberg, 2000) and in seawater (Wangberg et al., 2001; Mason et al., 2001; Rolfhus and Fitzgerald, 2001; Lanzillotta and Ferrara, 2001; Lanzillotta et al., 2002). The occurrence of the highest DGM concentration values at the daily interval of maximum insolation and in summer supports this thesis. Photo-reduction may occur by photo-chemical reactions (Nriagu, 1994) in which organic substances take part (Matthiessen, 1998; Lanzillotta et al., 2004; O'Driscoll et al., 2004; Garcia et al., 2005), and/or by direct reactions of photolysis of the Hg^{2+} complexes to Hg^{0}. The presence of Dissolved Organic Matter (DOM) may consistently play a role in the reaction rate of DGM formation (Costa and Liss, 1999, 2000); in particular, it has been identified a photo-sensitising role of the humic substances in marine photochemical reactions

(Costa and Liss, 1999). However, Mason and Sullivan (1999) reported that mercury complexation by organic ligands reduces the concentration of Hg^{2+} available for the reduction processes. Besides an "abiotic" formation of DGM, a "biotic" Hg^{2+} reduction has been suggested to explain its presence in deep waters, especially close to the sediment (Kim and Fitzgerald, 1988). The importance of microorganisms on the direct formation of DGM under dark conditions has been suggested by some researches carried out in the Pacific Ocean; bacteria associated with deep marine sediments can produce Hg^0 which is transported to the surface in the upwelling zones by the water currents (Kim and Fitzgerald, 1988).

The capability of microorganisms to reduce mercury compounds to volatile mercury is due to the unique characteristics of this metal. In fact to be detoxified by reduction, the redox potential of a given heavy-metal should be between that of the hydrogen/proton couple (-421 mV) and that of the oxygen/hydrogen couple (+808 mV) [calculed from Weast (1984) at 30 °C and pH 7.0], which is the physiological redox range for most aerobic cells. Thus, Hg^{2+} (+430 mV) and Cu^{2+} (-268 mV) may be reduced by the cell, but Zn^{2+} (-1.18 V) and Cd^{2+} (-824 mV) may not. If a cell chooses to detoxifies a compound by reduction, an efflux system should be present to export the reduced products. Only in the case of mercury do reducibility and a lower vapour pressure of the metallic reduction fit together; mercury is thus detoxified by reduction of Hg^{2+} to Hg^{2+} with diffusional loss of the Hg^{2+}.

Nakamura et al. (2001) screened special mercury resistant bacteria in the mercury polluted sediments of the Minamata Bay (Japan), capable of volatilise mercury compounds at high rate, with the aim to develop a method for removing mercury by means of a biomass. Studies at high Hg^{2+} concentrations on the resistance to mercury of prokaryotic microorganisms have demonstrated that bacteria can convert both inorganic and organic mercury compounds to mercury vapour, using a plasmid-encoded enzymatic pathway, the *mer* gene; hence bacteria can play a role in the production of dissolved elemental mercury without the presence of solar

radiation (Barkay et al., 1991; Barkay, 2001). An interesting review of the bacterial resistance to mercury is reported by Barkey et al. (2003). The existence of enzymatic reduction processes of mercury acting in bacteria has been widely reported (Barkay and Wagner-Dobler, 2005). DGM production in the dark has been examined by Rolfhus and Fitzgerald (2004) adding Hg^{2+} to dark and light-incubated coastal seawater samples from Long Island Sound. The microbial reduction and oxidation of mercury in freshwaters lake were reported by Siciliano et al. (2002): authors concluded that H_2O_2 produced by solar radiation stimulates mercury oxidase activity in lake water, which results in a decrease in DGM levels during the afternoon.

Literature reports data indicating that eukaryotic microorganisms can also reduce mercury, but only a few papers report on direct measurements of cellular generation of volatile mercury species (Ben-Bassat et al., 1972; Mason et al., 1995). Ben-Bassat and Mayer (1977, 1978) and Bentz (1977) found that the formation of Hg^0 decreased as a function of the inhibition of photosynthesis in cultures of the green algae *Chlorella*. Moreover, the highest values of DGM concentration were observed together with the highest values of chlorophyll *a*, suggesting that phytoplankton may produce DGM (Vandal et al., 1991). It has been hypothesised that algae and diatoms are able to reduce Hg externally by cell surface enzymatic processes, like other metals (Jones et al., 1986). With refer to Cu^{2+} and Fe^{3+} and other metals, three mercury reduction path-ways were indentified for the diatom *Thalassiosira weissflogii*: reduction by organic compounds released into the medium, by cell wall components and via a plasmalemma enzymatic pathway (Price and Morel, 1990). Mason et al. (1995) found that small phytoplanktonic microorganisms (typically <3 μm diameter such as cyanobacteria) are the primary mercury reducers. Lanzillotta et al. (2004), studying the processes of DGM formation by phytoplanktonic microorganisms and by their organic compounds released into

the marine environment, concluded that DGM formation derives mainly from a photochemical process acting on the biogenic organic matter.

1.3. Toxicity of heavy metals

Of the 90 naturally occurring elements, 21 are non-metals, 16 are light metals and the remaining 53 are heavy metals (Weast, 1984).

Most heavy metals are transition elements with incompletely filled d orbitals. These d orbitals provide heavy-metal cations with the ability to be redox-active. Hence a number of trace metals are used by living organisms to stabilize protein structures, facilitate electron transfer reactions and catalyze enzymatic reactions. For example, copper (Cu), zinc (Zn) and iron (Fe) are essential as constituents of the catalytic sites of several enzymes. Other metals, such as lead (Pb), mercury (Hg) and cadmium (Cd) may displace or substitute for essential trace metals and interfere with proper functioning of enzymes and associated cofactors (Nies, 1999).

The main biological characteristics of the most common heavy metals are described in the following paragraphs.

1.3.1. Copper (Cu)

The electrochemical potential of Cu^{2+}/Cu^{+} is -268 mV, well within the physiological range. Copper easily interacts with radicals, best with molecular oxygen. Its radical character makes copper very toxic. Copper toxicity is based on the production of hydroperoxide radicals (Rodriguez Montelongo et al., 1993) and on interaction with the cell membrane (Suwalsky et al., 1998).

Besides copper/zinc superoxide dismutase, the most important function of copper is in the cytocrome *c* oxidase and related enzymes, which are oxygen-dependent terminal oxidasees in the respiratory chain of many organisms (Nies, 1999).

1.3.2. Zinc (Zn)

Zinc occurs exclusively as the divalent cation Zn^{2+}. With its completely filled d orbitals, the zinc cation is not able to undergo redox changes under biological conditions. Zinc is a component in such a variety of enzymes and DNA-binding proteins, such as zinc-finger proteins, which also exist in bacteria (Chou et al., 1998), that life seems not to be possible without this redox-inactive element. Zinc may be complexed by various cellular components and is transported by members of a variety of protein families (Nies, 1999).

1.3.3. Lead (Pb)

Pb appears ubiquitous in aquatic ecosystems and is bioaccumulated in aquatic organisms (Moriarty, 1990). It occurs in the environment in a wide range of physical and chemical forms that greatly influence its behaviour and its effects on the ecosystem. Most of the Pb in the environment is in the inorganic form and exists in several oxidation states (0, I, II, and IV). According to Nussey et al. (2000), Pb(II) is the most stable ionic species present in the environment and is thought to be the form in which most Pb is bioaccumulated by aquatic organisms (Jackson et al., 2005). Lead has been used in large amounts for 2500 years (Hong et al., 1994), recently as a fuel additive, although the toxicity of lead for animals and man has been well known for a long time (Johnson, 1998). Lead acts on the central nervous system, on blood pressure and on reproduction (Goyer, 1993). Pb resistance seems to be based predominantly on metal ion efflux (Nies, 1999).

1.3.4. Cadmium (Cd)

A consistent amount of work has been done especially on cadmium toxicity in microorganisms; its effect regard the "thiol-binding and protein denaturation", the "interaction with calcium metabolism and membrane damage" and the "interaction with zinc metabolism". The main detoxification systems acting in plant and algae seems to be mediated by transport of glutathione/phytochelatin complexes by proteic transporters into the vacuoles (Nies, 1999).

1.3.5. Mercury (Hg)

Mercury is the most toxic of all the heavy metals. Both dissolved, inorganic Hg and MeHg, accumulate in phytoplankton (Andren et al., 1998) by passive diffusion across the membrane (Mason et al., 1995) or by facilitated diffusion transport (Watras et al., 1998). However, in contrast to MeHg, inorganic Hg is not biomagnified in the trophic transfer from phytoplankton to zooplankton. MeHg reaches its highest concentration in the tissues of fish at the top of aquatic food chain (WHO,1989). The affinity of Hg^{2+} to thiol groups is even stronger than the affinity of cadmium to sulphide.

Resistance to mercury is based on its unique peculiarities: its redox potential [its electrochemical potential of Hg^{2+}/Hg^{0} at pH 7 is +430 mV] and the vapour pressure/melting/boiling point of metallic mercury, which is extraordinarily low for a metal [melting point -39 °C, boiling point 357 °C (Weast, 1984)]. Thus living cells such as bacteria are able to reduce Hg^{2+} to Hg^{0}, which does not remain inside the cell with the potential of becoming oxidized again, but leaves the cell by passive diffusion (Silver, 1996; Silver and Phung, 1996). Once outside, however, metallic mercury may be oxidized again by other bacteria (Smith et al., 1998).

1.3.6. Effect of heavy metals on autotrophic protists

As regards autotrophic protists, previous works have showed that toxic metals act on photosynthesis (Rosko and Rachlin, 1977), cell division (Rachlin et al., 1983), membrane permeability (Rachlin and Grosso, 1993) and cell motility (Anderson and Morel, 1978; Fennikoh et al., 1978).

To have any physiological or toxic effect, metal ions have to enter the cell. Surfaces of living particles are characterized by various sites capable to adsorb metal ions with high affinity (Scarano and Morelli, 1993). The binding of metals to the cell surface represents the initial mechanism of the metal ion uptake process of the microorganisms. When metal ions equilibrate on the outside of the cell, this rapid step is followed by a relatively slow uptake due to the membrane transport of the metal inside the cell. The adsorption process on cell surface can be interpreted in terms of surface coordination between the metal and one or more functional groups on the cell surface (Scarano and Morelli, 1993). The final equilibrium of this process depends on the chemical composition of the surrounding solution. Once inside the cell, metals affect cellular metabolism by forming coordination complexes with various biomolecules, including numerous enzymes that require specific metals as essential cofactors. However, when in excess, metal ions can interfere with numerous physiological processes, thus resulting very toxic to the cells. Hence, the intracellular concentration of heavy-metal ions has to be tightly controlled by living organisms.

Numerous species of algae, both macro- and micro-algae, are capable of sequestering significant quantities of either nutrient or toxic heavy metal ions from aqueous solutions. In order to cope with metal toxicity, the following mechanisms of detoxification have been observed in algae and autotrophic microorganisms (Gonzales-Davila, 1995):

1) the development of energy driven-efflux pumps that keep toxic element levels low in the interior of the cell.

2) Oxidation state change by which a more toxic form of a metal can enzymatically and intracellularly be converted to a less toxic form.
3) Precipitation of insoluble metal complexes on the cell surface.
4) Complexing of metal ions with excreted metabolites (extracellular products), which can extracellularly mask a toxic metal.
5) Vaporization and elimination by means of converting a toxic metal to the volatile chemical species.
6) Binding of metal ions with proteins or polysaccharides in the interior of the cell, which may deactivate the metal ion's toxicity.
7) Methylation of the element, which can enzymatically and intacellularly prevent a toxic element from reacting with a –SH group.

1.4. Ecological monitoring of heavy metal pollution

According to Robinson and Avenant-Oldewage (1997) the two factors which contribute principally to the damaging effect of metals as environmental pollutants are, firstly, their inadequate biological degradation to inert metals (as in the case of most organic pollutants), and secondly, the propensity of metals to accumulate and to remain largely in the aquatic environment.

The assessment of pollution effects may be more difficult on the ecosystem level than on the level of single species. There are a great deal of data on metal concentrations in various organisms exposed to experimental contamination or taken from areas subjected to anthropogenic inputs of metals, so as many are the studies that, taking solely metal concentrations in waters into account, have determined sub-lethal or acute toxicological parameters. However, these data do not permit the establishment of direct relations between metal pollution and toxicity of metals. The concentrations in living organisms are not related

to the concentrations in water by a simple invariable function which takes no account of the chemistry of metals in seawater. When variations of the chemical composition of the external medium occur, numerous organisms are able to keep their internal chemical composition at a steady level, compatible with the normal development of their physiological functions. This ability varies from species to species. However, the ability to regulate is effective for moderate variations of concentrations in the environment. Moreover this ability seems to vary according to the physiological functions of the trace elements (Amiard-Triquet and Amiard, 1980). According to Robinson and Avenant-Oldewage (1997) and Hernandez-Hernandez et al. (1990), several factors affect the toxicity of pollutants to aquatic organisms and can be divided into biotic and abiotic factors. The former include physiological conditions, tolerance, growth and reproduction, species variations, inter- and intra-specific variation in life history stages, adaptive capabilities and behavioural responses; the latter could be represented by metal species in the water, the presence of other metals or pollutants, nature of dissolved organic matter, pH, temperature, alkalinity and hardness, metal interactions and dissolved oxygen and interactions between all them. The effect of two or more toxicants may be additive, antagonistic or even synergistic (Jackson et al., 2005). Hence, chemical analysis of individual toxic compounds are not adequate indicators of their associated biological and ecological effects and they should be combined with biological tools to assess the toxicity of the chemical that is biologically available (Martin-Diaz et al., 2004). Also, in addition to the common criteria on the individual level, such as mortality, reduced growth rates, metabolic activities and reproduction, further indications of stress may be of ecological significance. Toxicity measurement of wastewater, sediments and contaminated water bodies is a very important part of environmental pollution monitoring. Toxicity tests are desirable in water pollution evaluations because chemical and physical tests alone are not sufficient to assess potential effects on aquatic biota.

The effect of heavy metals and other toxicants on various animal taxa and life stages of them have been extensively reported in the literature (MacDonald et al., 1988; McKenney and Neff, 1979; Ober et al., 1987; Stark, 1998; Jackson et al., 2005).

Hernadez-Hernandez et al. (1990) stated that with regard to metal bioaccumulation in marine organisms, several authors have proved their high accumulation ability in crustaceans, mollusks and fishes, which generally depends on their exposure time and the concentrations of metals in the water. In the processes of transformation and accumulation of contaminants, microorganisms participate primarily due to their considerable amount in the ecosystems and their great adaptability (Nelson and Colwell, 1975); in particular marine microalgae are promising indicator species for organic and inorganic pollutants and constitute important tools to monitor physiological changes in the presence of heavy metals (Torres et al., 2008).

1.4.1. Sediment toxicity tests

The majority of chemicals discharged into aquatic system eventually end up in sediments that may act both as sink and source of pollution. Many toxic and bioaccumulative pollutants are found only in trace amounts in water and often at elevated levels in sediments. In fact,the highest accumulation factors of all potential sample materials of the marine environment is exhibited by sediments and especially by the finest grain size fractions. Sediments near urban areas commonly contain high levels of contaminants, constituting a major environmental problem faced by many anthropogenically impacted aquatic environments.

The role of aquatic sediments as a sink and source of large quantities of contaminants has led to the development of a wide variety of bioassays for the toxicological assessment of sediments; they represent complementary approaches for characterizing the biological effects and hazards of contaminated sediments. These procedures range in complexity from short-term lethality tests that measure effects of individual contaminants on single species to long-

term tests that determine the effects of chemical mixtures on the structure and function of communities.

The evaluated sediment phase may include whole sediment, suspended sediment, elutriates, or sediment extracts. Although whole-sediment testing seems the most realistic approach to evaluate the bioavailability of contaminants in sediments, porewaters and aqueous extracts are frequently used. Elutriate, consisting of seawater re-suspension of sediments, provides a measure of the amount of a substance that is exchanged between the sediment and the aqueous phase during sediment disturbance and gives information on the leaching capability of sediment-associated contaminant.

Elutriate Sediment Toxicity Test (ESTT) have been extensively considered the best method to replicate disturbance effects on the release of chemicals from the sediment (US-EPA, 1991). The standard elutriate test was jointly developed in the early 1970s by the U.S. Army Corps of Engineers and the U.S. Environmental Protection Agency to monitor the soluble release of contaminants into the water column during open-water disposal of dredged sediments. This approach is used in sediment studies to simulate processes that might disturb the sediment and bring contaminants into the water column. Toxicity testing of sediment elutriates is important because dissolved forms of pollutants are more bioavailable to aquatic biota for uptake and are the primary cause of adverse impacts in aquatic ecosystems.

In the past two decades a wide variety of toxicological tests, involving organisms at different trophic levels, including bacteria, phytoplankton, mollusks, crustaceans and fishes has been developed in order to assess the biological effects of contaminants in sediments (Cheung et al., 1997; Matthiessen et al., 1998; Geffard et al., 2003; Arizzi-Novelli et al., 2006).

The release, the bioavailability and the toxicity of contaminants in elutriates of estuarine sediments have been examined by studying concurrently their effects both on the

embryogenesis and on the larval growth of the *Crassostrea gigas* larvae together with their bioaccumulation in those organisms (Mucha et al., 2004).

Several laboratory conditions for preparing and testing elutriates of sediments of industrial and urban contaminated areas of the Lagoon of Venice (Italy) have been assessed in experiments by using embryos of the sea urchin *Paracentrotus lividus* (Marin et al., 2001). The bioavailability of Cd, Cu, Zn, and Pb in the elutriates of two metal-contaminated sediments (Bidassoa and Dunkerque) has been studied by using the presence of metallothioneins in *Crassostrea gigas* larvae as a biomarker of metal exposure (Geffard et al., 2007); these authors concluded that the production of metallothioneins is a more sensitive indicator of heavy metal pollution than other physiological endpoints and could be proposed as an early biomarker of metal exposure in larvae.

Cheung et al. (1997) carried out Elutriate Sediment Toxicity Tests, using two microalgae *Skeletonema costatum* (a diatom) and *Dunaliella tertiolecta* (a green alga), juvenile shrimp (*Metapenaeus ensis*) and juvenile fish (*Trachinotus obtaus*), with the aim to study the feasibility of using different trophic organisms for evaluating the toxicity of dredged sediments arising in Hong Kong; these authors also employed two commercially available tests using bacteria (Microtox Test and Toxi-Chromotest) to test both the solid phase and elutriates of the sediments and concluded that bioassay tests using diatom on the sediment elutriate were correlated significantly ($p < 0.05$) with a number of physico-chemical properties of sediments and elutriates.

Bioassays with microorganisms are widely used as tools in estimating the potential risk of contaminated sediments. In particular, in the assessment of toxicity of sediment elutriates, bioassays based on the growth of phytoplanktonic microorganisms have been largely used (Pardos et al., 1998; Mucha et al., 2004; Tolun et al., 2001; Wong et al., 1999) because of their sensitivity to different contaminants. Davoren et al. (2005), using an integrated approach

based on the use of a number of bioassays representing multiple trophic levels, concluded that the algal test was the most responsive to elutriates of estuarine sediments.

1.4.2. Biomarkers

The presence of chemical compounds in natural aquatic systems does not indicate, by itself, injurious effects to organisms (Wang et al., 1998), as bioavailability of these compounds should also be taken into account. The use of biomarkers can be an important tool for evaluating toxic effects of bioavailable contaminants.

A biomarker may be defined as a biochemical variation measured in tissue/body fluids of an organism that provides evidence of exposure and effects of one or more chemical pollutants (Phillips and Rainbow 1993; Depledge and Fossi 1994).

Biological monitoring or biomonitoring can be defined as the systematic use of biological responses to evaluate changes in the environment, with the intent of establishing a quality control program (Cairns and van der Schalie, 1980). Typically, biomarkers are considered quantitative measures of changes in the biological system that can be related to exposure to the toxic effects of environmental chemicals (WHO, 1993; Peakall and Walker, 1994). Although not explicitly contained in most definitions, the use of the term "biomarker" or "biomarker response" is often restricted to cellular, biochemical, molecular, or physiological changes that are measured in cells, body fluids, tissues, or organs within an organism that are indicative of xenobiotic exposure (Van Gestel and Van Brummelen, 1996; van der Oost et al., 2003; Lam and Gray, 2003). Often pollutant exposure at contaminated sites does not result in lethality to resident biota, but may produce more sub-lethal effects that may compromise the biochemical, physiological and reproductive functions of living organisms, and, ultimately, influence the long-term survival of populations. The advantages of biochemical biomarkers are that: 1) they represent a direct biological response to pollutant exposure; 2) they often

respond to exposure at concentrations lower than that required for effects at the individual or population level; 3) they are often induced prior to effects at higher organizational levels. Thereby they constitute an early warning indication of the toxicity of a pollutant (MacFarlane et al., 2006; Torres et al., 2008).

In recent years a great variety of organisms have been employed in biomonitoring programs in order to assess the impact of pollutants on the aquatic environment. Bivalve mollusks, particularly mussels, have been elected as "sentinel" organisms in international environmental monitoring programs as part of the MUSSEL WATCH PROGRAM (Goldberg, 1975; Tavares et al., 1988; Claisse, 1989; Tripp et al., 1992; Tanabe, 1994). Many other organisms have also been used as regionally important tools in environmental programs, e.g., mangrove mussels in South Brazil (Torres et al., 2002), crabs in South Africa (Thawley et al., 2004), polychaetes in Spain and France (Gesteira and Dauvin, 2000), fish in Australia, Asia, and America (Edwards et al., 2001; Ueno et al., 2005; Carrasco-Letelier et al., 2006), respectively.

In addition to the massive use of marine animals in biomonitoring programs, photosynthetic organisms like algae have increasingly been used as biodetectors to monitor xenobiotics in marine environments (Jayasekera and Rossbach, 1996; Ali et al., 1999; Sánchez-Rodríguez et al., 2001; Barreiro et al., 2002; Conti and Cecchetti, 2003; Conti et al., 2007). Because of their natural and widespread occurrence along worldwide seashores, photosynthesizing organisms could be useful for a time-integrated picture of the ecosystem response to exposure to toxic compounds. Physiological changes, both in macroalgae (Sánchez-Rodríguez et al., 2001; Conti and Cecchetti, 2003) and microalgae (Rijstenbil et al., 1994; Tripathi et al., 2006), are important tools in the hazard of heavy metals in the aquatic environment (Torres et al., 2008). Algae have been suggested and used as potential bioindicators of aquatic pollution and its metabolic response to xenobiotic could point to important biomarkers (Witton and Kelly, 1995; Ali et al., 1999). Moreover, autotrophic microorganisms are particularly promising

indicator species for organic and inorganic pollutants since they are typically the most abundant life forms in aquatic environments and occupy the base of the food chain. The study of physiological and biochemical alterations, as well as the identification and quantification of pollutants in basal-level trophic organisms are an essential diagnostic tool (Van Gestel and Van Brummelen, 1996, Handy et al., 2003).

The presence of metals in the plant kingdom induces the synthesis of several proteins, mainly phytochelatins (PC) (Cobbett and Goldsbrough, 2002; Perales-Vela et al., 2006), but also metallothionein (MTs) (Vasak, 2005), and heat shock proteins (HSPs) (Spijkerman et al., 2007).

Metallothioniens and phytochelatins are similar in many ways, including the high number of cysteine molecules, and the fact that both are responsible for the detoxification of heavy metals. In fact, PC were originally classified as class 3 MTs, until they were deemed sufficiently different in structure and synthesis pathway to be classified as PC. All MTs have three characteristics in common: they have low molecular weight (6–7 kDa), a large fraction of cysteine residues, and a high metal content with coordination of metal ions in metal-thiolate clusters. Metallothioneins, cysteine-rich and metal-binding proteins, are products of mRNA translation and this distinguishes them from PC, which are the product of an enzymatic synthesis (Grill et al., 1989; Cobbett and Goldsbrough, 2002).

The presence of intracellular PC constitutes an early and specific signal of metal stress in plants and autotrophic microorganisms (Cobbett, 2000; Kawakami et al., 2006a), so these compounds can be considered suitable biochemical indicators of metal exposure.

1.4.2.1. Phytochelatins

A widespread mechanism of defence developed by plants and autotrophic microorganisms against metal stress involves phytochelatins. The general structure has been determined to be

(γ-Glu-Cys)$_n$-Gly where chain length "*n*" ranges between 2 and 11 units (Rauser, 1995, Steffens, 1986, Cobbett and Goldsbrough, 2002). It is important to note that the glutammic acid residues are not bond with cysteine by means of an α-carboxyl group as in transcriptional amminoacids but with an γ-carboxyl group. The gamma-glutammyl linkages present, which cannot be prepared by ribosomes, lead to the search for an enzyme-mediated path for the production of PC. Grill et al., 1989 demonstrated that PC are synthesized by the enzyme "phytochelatin synthase" (PC), which is a γ-glutamylcysteine dipeptidyl transpeptidase. It catalyzes the transpeptidation of the γ-Glu–Cys moiety of glutathione (γECG) onto a second γECG molecule to form PC_2 or onto a PC molecule to produce a $n + 1$ oligomer. The enzyme was described as a tetramer of MW 95 000 with a Km for glutathione of 6.7 mM (Steffens, 1986 and Cobbett and Goldsbrough, 2002).

The general mechanism involved in PC biosynthesis is:

$$\mathbf{[\gamma Glu–Cys]_{\mathit{n}}\text{-}Gly + [\gamma Glu–Cys]\text{-}Gly \rightarrow [\gamma Glu–Cys]_{\mathit{n}+1}\text{-}Gly + Gly}$$

Numerous physiological, biochemical and genetic studies have confirmed that glutathione (or, in some cases, related compounds) is the substrate for PC biosynthesis (Rauser, 1995, 1999; Zenk, 1996). Early studies with cell cultures demonstrated that induction of PC in the presence of Cd coincided with a transient decrease in levels of glutathione. Furthermore, the exposure of either cell cultures or intact plants to an inhibitor of glutathione biosynthesis, "buthionine sulfoximine", conferred increased sensitivity to Cd with a corresponding inhibition of PC biosynthesis. This could be reversed by the addition of glutathione to the growth medium.

The dependence of phytochelatin synthase on heavy metals for activity has invariably been interpreted in terms of direct metal binding to the enzyme. Few investigators have considered

explicitly how heavy metals activate PC synthase but those that have considered it, have assumed that activation is consequent on the direct binding of metal ions to the enzyme (Zenk,1996; Cobbett, 2000). Indeed, in the most recent model for PC synthase action, it has been proposed that the strongly conserved N-terminal half of the enzyme is responsible for catalysis and that activation arises from the binding of metal ions to residues, possibly cysteine residues, within this domain. This provides a mechanism to autoregulate the biosynthesis of PC in which the product of the reaction chelates the activating metal, thereby terminating the reaction (Cobbett, 2000, Fig. 1.3).

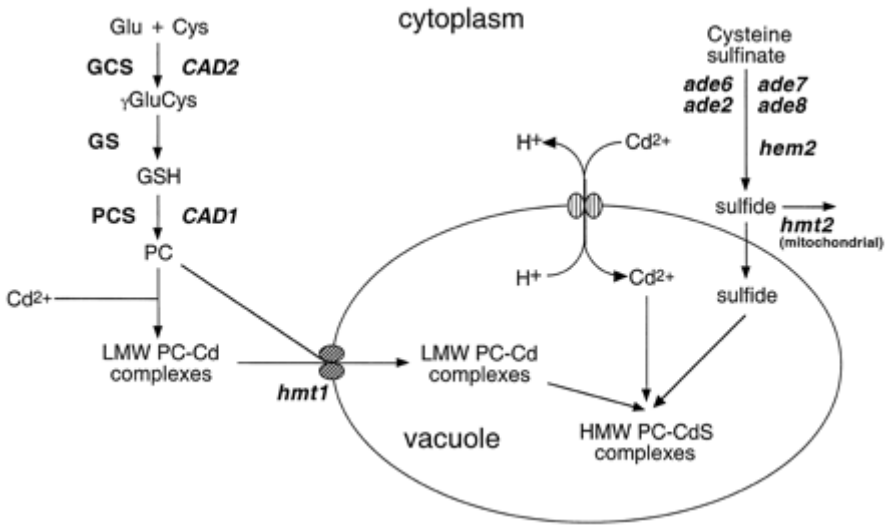

Fig. 1.3. PC biosynthetic pathway (Cobbett, 2000).

In the last years a considerable interest has been attracted to PC as biochemical indicators of metal stress both in higher plants (Keltjens et al., 1998; Sneller et al., 1999; Sun et al., 2005) and in microalgae (Ahner et al., 1997; Knauer et al., 1998; Pawlik-Skowronska, 2000, Wei et al., 2003; Le Faucheur et al., 2005a), since these compounds constitute an early and specific signal of the actual intracellular metal concentration. This is supported by the finding that enhanced cellular PC have been measured in natural populations of marine and freshwater

microalgae collected in polluted areas (Ahner et al., 1994; Ahner et al., 1997; Knauer et al., 1998).

The induction of phytochelatins in phytoplanktonic algae has been demonstrated, both in laboratory cultures (Gekeler et al., 1988) and in field studies (Ahner et al., 1997), even at very low concentrations of many heavy metals. Although a direct relationship between metal dose and PC production has been observed, the magnitude of the response, the degree of polymerization of PC synthesized and the relative level of individual peptides seem to depend on the organism analyzed (Ahner et al., 1995) as well as on the particular metal used as inducer (Ahner and Morel, 1995). Constitutive differences such as the size of the cellular glutathione pool, the rate of metal uptake or the capability to activate other mechanisms of metal tolerance, can account for the wide variability of PC production among species.

CHAPTER 2

AIM OF THE STUDY

The research performed in the framework of the PhD course has been devoted to analyse the workings of known biological metal-resistance systems and their ecological significance with the aim to give a contribution to the comprehension of the processes of transport, transformation and accumulation of heavy metals in the marine environment.

Metal ions, regardless of whether they are biologically essential or not, may exert toxic effects to the living organisms when a critical concentration is reached. This concentration depends on the organism, on the metal and on the metal speciation. In aquatic systems, metal ions or compounds in solution are available for biota by adsorption on the surface of organisms and by translocation into the cells.

Many organisms can grow in contaminated environments since they have developed physiological adaptations to metal excess as defence mechanism. Among others, unicellular phytoplankton possess molecular mechanisms that allow them to discriminate non-essential heavy metals from the essential ones for their growth. In addition, they must maintain non-toxic concentrations of these ions inside their cells. In this way, two principal mechanisms have been identified, one which prevents the indiscriminate entrance of metal ions into the cell, i.e., exclusion, and the other which prevents bioavailability of these toxic ions once inside the cell, i.e., the formation of complexes and the metal volatilization.

On the basis of the simplicity of metal exposure and because the same cells both absorb and detoxify metal ions, unicellular phytoplankton represent suitable organisms for the purpose of surveilling and improving water quality, constituting sensitive indicators of the metal load of aquatic ecosystems. Moreover autotrophic microorganisms form the base of marine food web

and it is obvious that, through the potential transfer along food chains, they might play a fundamental role in accumulation of heavy metals.

The goal of this research was to evaluate and to compare the ecological importance of the detoxification processes executed by different taxa of autotrophic protists exposed to environmentally relevant levels of dissolved Cd, Cu, Pb, Zn and Hg. In addition, a feature of this research was devoted to examine the detoxification mechanisms acting in these microorganisms, which can be suitable to investigate the quality of metal-polluted water systems.

Mercury experiments needed a separate study given the chemical particularities of this metal in comparison to the other heavy metals and the peculiarity of the experimental methodology necessary for mercury determination.

It is well known that a widespread mechanism of defence developed by plants and algae against metal stress involves intracellular metal-binding peptides. These molecules, called phytochelatins (PC), have glutathione (GSH) as biosynthetic precursor, and their main function in cells is to chelate metal ions in the cytoplasm, thereby reducing the concentration of the cytotoxic free metal ions. Five species of autotrophic protists: *Phaeodactylum tricornutum*, *Thalassiosira weissflogii* and *Skeletonema costatum* (diatoms), *Dunaliella tertiolecta*, (green algae), *Emiliania huxleyi* (coccolitophore), were examined in order to understand if this defence mechanism is conservative throughout the species.

Initially the research was focalized on the evaluation of the suitability to use the accumulation of PC as a biomarker of metal bioavailability in bioassays for the assessment of metal pollution in the marine environment. Results of preliminary laboratory experiments, carried out with *P. tricornutum* cells incubated in EDTA-buffered artificial seawater added with known amount of Cd, Pb and Cu metal ions, showed increasing cellular PC concentration with increasing free metal ions in the medium, indicating that PC behave as a biomarker of

exposure to the bioavailable metal fraction. Comparative experiments carried out using controlled systems, such as laboratory cultures of the five species of eukaryotic microorganisms, showed that *Phaeodactylum tricornutum*, *Thalassiosira weissflogii* and *Skeletonema costatum* were the more sensitive species with respect to the synthesis of PC. So, these diatoms were used to develop a new bioassay involving the presence of PC as response to metal bioavailability in re-suspensions of marine sediments (elutriates) collected in a metal-polluted coastal area (Foce dello Scolmatore and Marina di Pisa). Elutriates of marine sediments were considered because they yields useful information on the metal burden of natural waters. In fact sediments represent the major sink for contaminants in aquatic systems and subsequently sediment re-suspension can act as source of contaminants for the overlying water column (Forstner and Salomons, 1991).

Successively, *T. weissflogii* was investigated in order to understand the relative importance of two defence mechanisms acting in this diatom when exposed to potentially toxic concentration of mercury. Besides the ability to synthesize metal binding peptides, its capability to produce dissolved gaseous mercury (DGM) was investigated. In the literature there are many reports concerning the synthesis of PC in eukaryotic microalgae in response to heavy metals, such as Cd and at lesser extent Cu, Pb or Zn, but only a very few studies report on the use of Hg as a PC inductor. As regards the DGM production in aquatic systems, the existence of enzymatic reduction processes of mercury acting in bacteria has been widely reported (Barkay et al., 1991; Barkay, 2001; Barkey et al., 2003; Barkay and Wagner-Dobler, 2005; Nakamura et al., 2001); only a few papers assumed the existence of DGM production by eukaryotic microorganisms, although this mechanism is poorly known (Ben-Bassat and Mayer, 1977,1978; Bentz, 1977; Mason et al., 1995). To our knowledge, no attempt has been made to follow and correlate both these Hg-induced responses.

The ecological importance of the process of DGM production in the context of the biogeochemical cycle of this metal has been also discussed. In aquatic systems the DGM formation and its volatilization to the atmosphere is a phenomenon comparable to terrestrial emissions (Lindberg et al., 1998; Gardfeldt et al., 2003); moreover, through DGM production, mercury is removed from the water column and is no longer available for methylation processes and subsequent bioaccumulation throughout food chains.

CHAPTER 3

METHODOLOGY

3.1. Laboratory cultures of marine autotrophic protists

The marine autotrophic protists: *Phaeodactylum tricornutum* (Bohlin), *Thalassiosira weissflogii* (Grunow in Van Hemck) Fryxell & Haxle and *Skeletonema costatum* (Greville) Cleve (diatoms), *Dunaliella tertiolecta*, (green algae), *Emiliania huxleyi* (coccolithophorid), were obtained from the Culture Collection of Algae and Protozoa, Dunstaffnage Marine Laboratory, UK.

Cellular volumes were estimated from cell measurements and assumption of particular geometric shapes (Tab. 3.1).

Strain		Shape	Volume (μm^3)	S.D.
Thalassiosira weissflogii	(CCAP 1085/1)	Cylinder	1247	± 616
Phaeodactylum tricornutum	(CCAP 1052/1A)	double cone	168	± 31
Skeletonema costatum	(CCAP 1077/5)	Cylinder	323	± 120
Dunaliella tertiolecta	(CCAP 19/27)	prolate spheroid	208	± 82
Emiliania huxleyi	(CCAP 920/3)	Sphere	101	± 31

Tab. 3.1. **S**train, shape and cellular volume of the microorganisms examined.

3.2. Culture conditions

Stock cultures of *P. tricornutum*, *T. weissflogii, S. costatum*, *D. tertiolecta* and *E. huxleyi*, were grown in axenic conditions, in natural seawater enriched with f/2 medium (Guillard, 1975) at one–fifth the normal trace metal concentration (Tab. 3.2), at 21°C and fluorescent daylight (100 μmol photons × m^{-2} × s^{-1}) in a 16:8 light-dark cycle. The culture medium was sterilized using a filtration set with 0.2 μm sterile membrane filters (Sartorius), under a

camera-box with laminar airflow. Exponential growth was maintained by inoculating weekly into a fresh sterilized medium.

Nutrients	Molar concentration
$NaNO_3$	$8.8 \cdot 10^{-4}$
$NaH_2PO_4 \cdot H_2O$	$3.63 \cdot 10^{-5}$
$Na_2SiO_3 \cdot 9H_2O$	$5 \cdot 10^{-5}$
$CuSO_4 \cdot 5H_2O$	$8 \cdot 10^{-9}$
$ZnSO_4 \cdot 7H_2O$	$1.53 \cdot 10^{-8}$
$MnCl_2 \cdot 4H_2O$	$1.8 \cdot 10^{-7}$
$Na_2MoO_4 \cdot 2H_2O$	$5.2 \cdot 10^{-9}$
$CoCl_2 \cdot 6H2O$	$8.4 \cdot 10^{-9}$
$FeCl_3 \cdot 6H_2O$	$2.3 \cdot 10^{-6}$
EDTA	$2.3 \cdot 10^{-6}$
Vitamin B12	0.01 μg L^{-1}
Biotin	0.01 μg L^{-1}
Tiamin	200 μg L^{-1}

Tab. 3.2. f/2 medium (Guillard, 1975) modified at one–fifth the normal trace metal concentration.

3.3. Cell density measurement

Cell density was measured, under an optical microscope (Zeiss), by means of two types of haemocytometers (depending on cellular density): the Thoma counting chamber and the Neubauer counting chamber.

In both haemocytometers the slide consists of two sets of grids for replicates.

For as regards the Thoma counting chamber each grid consist of 16 fields containing 256 squares; the area of each square is 0.0025 mm^2, corresponding to a total area of each grid of 0.64 mm^2 and to a volume of $64 \cdot 10^{-6}$ cm^3 (chamber depth = 0.01 cm). Cell count consists of the total number of cells found in the 16 fields; cell density (cells mL^{-1}) is obtained using the following proportion:

- Average counted cells : $64 \cdot 10^{-6}$ cm^3 = x* : 1 cm^3 (*x = cell number)

For as regards the Neubauer counting chamber, the cell count consists of the total number of cells found in the 4 corner fields of the grid. The total area of the 4 corner fields is 0.04 cm^2 and the volume is $4 \cdot 10^{-4}$ cm^3 (chamber depth = 0.01 cm). Cell density (cells mL^{-1}) is obtained using the following proportion:

- Average counted cells : $4 \cdot 10^{-4}$ cm^3 = x* : 1 cm^3 (*x = cell number)

3.4. Growth rate measurement

The growth rate of the cultures (μ), expressed as doublings $\cdot$ day^{-1}, has been evaluated during the exponential phase of growth by counting cells at the time of the inoculum (t_0) and after 6 days; the following equation has been used:

$$\mu = 1/t \log_2 (N/N_0)$$

t = days of growth

N_0= number of cells at t = 0

N= number of cells at t = 6

3.5. Natural seawater

Natural seawater, used throughout the research work, was collected 3 miles offshore from the Island of Capraia (Tyrrhenian Sea, Italy), filtered through 0.45 μm membrane filters (Sartorius) and stored in the dark at + 4 °C. To avoid any contamination, pretreatment and cleaning procedures for sampling bottles and other labware were performed following indications suggested by Mart (1976).

3.6. EDTA-buffered artificial seawater

EDTA-buffered artificial seawater was prepared by following the Aquil recipe (Price et al., 1991) omitting the micronutrient metal stock solution, but adding EDTA (10.0 μM) and calculated amounts of Cd, Pb or Cu. Free metal ion concentrations in these metal buffers were calculated by means of the MINEQL+ chemical equilibrium program (Westall et al., 1976). All solutions were allowed to equilibrate overnight before performing tests.

3.7. Sediment sampling and elutriate preparation

In the assessment of toxicity of sediment elutriates sediment samples were collected in a coastal area of the province of Pisa, Tuscany (Italy), affected by anthropogenic and industrial inputs. Two sediment samples, collected in the shore(line) on the northern side of the mouth of the Filling Channel of the Arno River (sediment A) and at Marina di Pisa (sediment B), were chosen for the development of the bioassay (Fig. 3.1).

In addition, sediment samples were collected approximately 500 m away from the shoreline, along a transect of twelve stations going from the northern side of the mouth of the Arno River (st 1) to the southern side of the mouth of the Serchio River (st 12), an area impacted by the plume of the Arno River (Vignudelli et al., 2004).

Surface sandy sediment samples (5-10 cm) were collected with a stainless steel Van Veen grab. The sediments were placed in acid-cleaned (10% HNO_3) polyethylene containers, transported to the laboratory as soon as possible, homogenized with a blender and stored at 4°C in the dark until their use. The elutriation procedure was carried out within 2 weeks after collection. An aliquot of the wet sediment was dried at 60° C to allow the ratio dry weight: fresh weight to be determined.

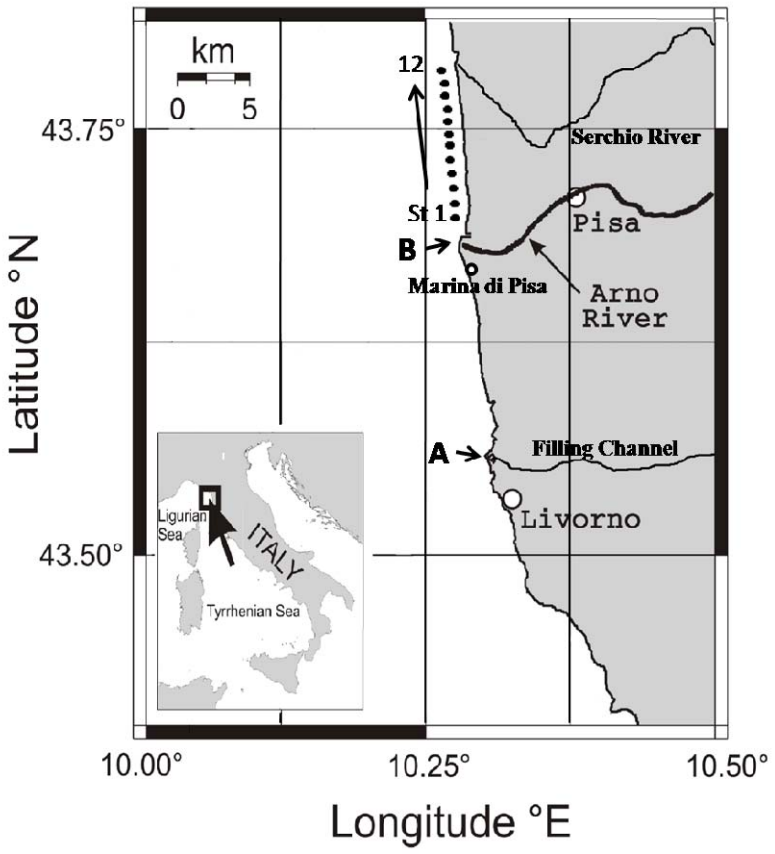

Fig. 3.1. Map of the two sites (named A and B) in the polluted coastal area in the province of Pisa (Tuscany, Italy) where sediments were collected.

The elutriation procedure was carried out mixing sediment to natural seawater in 1:4 (w/v) ratio, based on the sediment dry weight. The mixture was shaken at 500 rpm for 24 h at room temperature by using a vertical Stirrer (Velp Scientific) and let to settle for 30 min. The

aqueous fraction was centrifuged at 7000 rpm for 15 min and filtered (0.45 μm membrane filters). Elutriates were immediately used for bioassays or, alternatively, stored at -20° C.

3.8. Incubation experiments

3.8.1. Evaluation of phytochelatins in P. tricornutum as biomarker of metal exposure in marine waters

A first set of exposure experiments was carried out by using the marine diatom *P. tricornutum*. The capability of this diatom to synthesize phytochelatins in response to metal exposure has been widely studied by the research group working in the "Institute of Biophisics" of the CNR of Pisa (Morelli and Scarano, 2001; Morelli et al., 2002; Morelli and Scarano, 2004; Morelli and Fantozzi, 2008).

Before metal incubation experiments, a preculture was prepared by inoculating *P.tricornutum* from a stock culture on day 7 of growth (exponential phase) to provide an initial cell concentration of 5 - 7 × 10^4 cells mL^{-1} in natural seawater enriched with $NaNO_3$ and NaH_2PO_4 at a final concentration of 8.8 × 10^{-4} M and 3.6 × 10^{-5} M, respectively (equivalent to N and P concentrations in f/2 medium). In this medium, the growth rate of *P. tricornutum* was similar (10-15% lower) to that obtained in the maintenance medium. At the end of the logarithmic growth phase (approx. 2 × 10^6 cells mL^{-1}), calculated aliquots of the preculture were reduced to a volume of 10-20 mL by gentle filtration (1.2 μm membrane filters) and the resulting concentrated cell suspension was immediately transferred to the medium for PC induction experiments. The microorganisms were not let dry on the filter in order to avoid cell stress. Incubations were carried out both in EDTA-buffered artificial seawater and in natural seawater. Unless otherwise specified, 2 × 10^8 cells were incubated in 200 mL medium, to obtain a cell density of 1 × 10^6 cells mL^{-1}. PC induction experiments were carried out under continuous light conditions (100 μmol photons × m^{-2} × s^{-1}), at 21 °C. Cell counts carried out

after metal exposure showed that cell density was not appreciably changed during the incubation.

Before performing cell incubations, all the natural seawater samples were treated with $NaNO_3$ and NaH_2PO_4 at a final concentration equivalent to that of the preculture. This procedure was chosen to avoid the cells undergo significant changes in the culture medium. Metal enriched natural seawater samples were prepared by adding increasing amounts of Cd in the range 5 – 100 nM and of Pb or Cu in the range 25 – 200 nM. After 16 h equilibration and before cell addition, the electrochemically labile fraction of dissolved metal ions (Melab) was measured, at natural pH (8.2), by Anodic Stripping Voltammetry.

Seawater samples for field experiments were collected at six different stations, selected in contaminated areas in the province of the industrial city of Livorno, located in the Tyrrhenian coast of Tuscany (see map of Fig. 3.2).

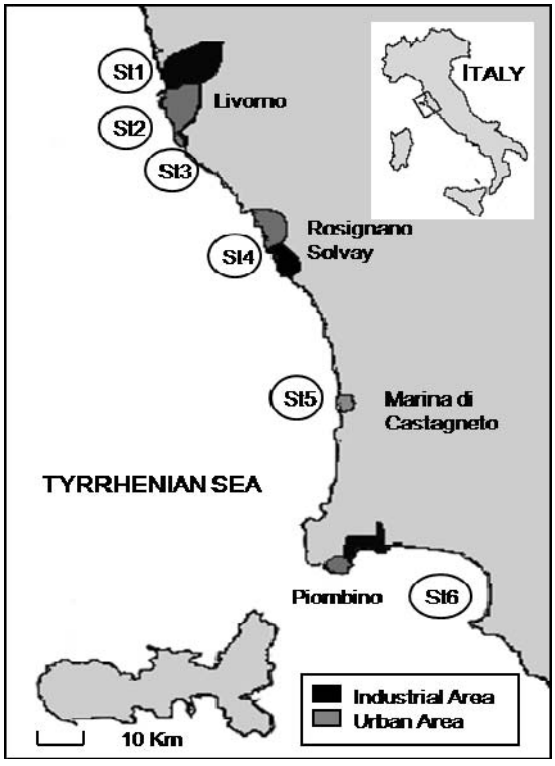

Fig. 3.2. Map of the province of Livorno where seawater samples were collected.

Tests were carried out by incubating 4×10^8 cells in a 1-liter sample (4×10^5 cells mL^{-1}) for 6 hours.

At the end of the exposure, cells were collected and submitted to the phytochelatins assay.

3.8.2. Comparison of PC response in different taxa of autotrophic protists

In order to compare the PC response in different taxa of autotrophic protists, short-term exposure experiments (6 h) were carried out by incubating the protists in natural seawater enriched with known amounts of heavy metals and assaying for phytochelatins. For this purpose, precultures were prepared by inoculating *P. tricornutum, S. costatum, T. weissflogii, D. tertiolecta and Emiliania huxleyi* from stock cultures on day 7 of growth (exponential phase) to provide initial cell densities ranging from 3-5 $\times 10^3$ cells mL^{-1} for *T. weissflogii*, to 1-5 $\times 10^4$ cells mL^{-1} for the other species.

The preparation of the precultures and the conditions of exposure were carried out accordingly to the procedure described for *P. tricornutum* in the previous paragraph.

The cell densities were: 5×10^5 cells mL^{-1} for *P. tricornutum,* 3×10^5 cells mL^{-1} for *S. costatum*, 4×10^4 cells mL^{-1} for *T. weissflogii*, 5×10^5 cells mL^{-1} for *D. tertiolecta* and 2×10^5 cells mL^{-1} for *Emiliania huxleyi*.

3.8.3. Evaluation of PC synthesis in P. tricornutum, T. weissflogii and S. costatum as biomarker of metal bioavailability in sediments

Two different experiments were carried out in order to evaluate the PC synthesis in *P. tricornutum*, *T. weissflogii* and *S. costatum* as biomarker of metal bioavailability in sediments.

In short-term incubation experiments, a preculture was prepared by inoculating cells from a stock culture on day 7 of growth (exponential phase) to provide an initial cell concentration of approximately 5×10^4 cells mL^{-1} for *P. tricornutum* and *S. costatum* and of 5×10^3 cells mL^{-}

[1] for *T. weissflogii*, in natural seawater enriched with the f/2 medium lacking the trace metal stock solution. At the end of the logarithmic growth phase, calculated aliquots of the preculture were reduced to a volume ≤ 10 mL by gentle filtration (1.2 μm membrane filters) and the resulting concentrated cell suspension was immediately transferred to the medium (200 mL) for PC induction experiments. Incubations were carried out under continuous light conditions, at 21 °C, for 5 h, using as media the elutriates diluted with natural seawater at 0, 25 , 50, 75 and 100% concentration. Cell densities were: 5×10^5 cells mL^{-1} for *P. tricornutum*, 4×10^4 cells mL^{-1} for *T. weissflogii*, and 3×10^5 cells mL^{-1} for *S. costatum*.

Long-term incubation experiments were carried out by inoculating cells from a stock culture (on day 7 of growth) in the elutriates properly diluted with natural seawater (from 0 to 100% concentration), at an initial cell density of 1×10^4 cells mL^{-1} for *P. tricornutum*, 1×10^3 cells mL^{-1} for *T. weissflogii*, and 5×10^3 cells mL^{-1} for *S. costatum*.

All media (100 mL) were enriched with the f/2 medium lacking the trace metal stock solution, and the cultures were let to grow during the exponential phase, for 6 days. Cell counts were performed at the 3rd and at the 6th day and the growth rate (expressed as doublings day^{-1}) was calculated.

3.8.4. Evaluation of phytochelatins synthesis and DGM production in T. weissflogii exposed to mercury

All the mercury incubation experiments were carried out using, as culture medium, natural seawater enriched with the f/2 medium lacking the trace metal stock solution. Calculated volumes of the stock cultures of *T. weissflogii* were used as inoculum to obtain an initial cell density of 1×10^3 cells mL^{-1}.

In a first set of incubation experiments, designed to evaluate the effect of mercury on the growth rate of *T. weissflogii*, 100 mL culture media were spiked with $HgCl_2$ to final

concentrations ranging from 5 to 750 nM. The cultures were allowed to grow for 6 days during the exponential phase and the growth was monitored by counting cells.

Two different experiments were carried out, with the aim to investigate the pattern of the non-protein thiols pool under mercury exposure. In a 2-day exposure experiment, 1-L cultures were exposed to a range of $HgCl_2$ concentrations, from 5 to 150 nM. At the end of the exposure (cell density was 1-2 x 10^4 cells mL^{-1}), aliquots of 800 mL and 50 mL of each culture were used for the determination of the non protein thiols and of the intracellular mercury concentration ($[Hg]_{intr}$), respectively. In a 7-day exposure experiment, a 2-L culture was exposed to 150 nM $HgCl_2$ and, at selected time intervals, from 0 to 7 days, aliquots of 50 mL of the culture were sampled and used for the determination of the $[Hg]_{intr}$, as well as aliquots of the culture from 800 to 200 mL, depending on the cell density, were sampled and used for the determination of the nonprotein thiols. In the exposure experiments, a control culture (no Hg added) was always used.

The production of DGM has been measured in cultures of *T. weissflogii* during the exponential phase of growth. For this purpose, 500-mL culture medium was spiked with $HgCl_2$ to reach an initial concentration of 5 nM and let to equilibrate for 3 days, before cell addition. At the end of the equilibrium time, the concentration of total dissolved mercury was approximately 65% of the initial one. This procedure was chosen to avoid the elevated abiotic DGM production occurring within the first days after mercury addition, as shown in preliminary experiments. This procedure is in agreement with data reported by several authors (Xiao et al., 1994; Mason et al., 1995), which observed a significant formation of Hg^0 within the first hours after the addition of mercury in the medium, due to chemical reactions independent from the biotic contribution. After the inoculum of *T. weissflogii* cells, two aliquots of 50 mL of the culture were sampled at time intervals and used for the measurement

of the DGM production and for the determination of the cellular mercury concentration ($[Hg]_{cell}$), respectively.

An additional experiment of DGM production was performed by using *T. weissflogii* cells treated with formaldehyde according to the following procedure. Mercury-treated cells from 50 mL of a culture at the 4th day of growth (cell density = 4-5 x 10^4 cells mL^{-1}) were collected by filtration (1.2 μm) and re-suspended for 10 min in a solution of 1.6% formaldehyde in seawater. Afterwards, the formaldehyde-killed cells were collected by filtration, re-suspended again in their growth medium and submitted to the measurement of DGM production.

3.9. Determination of phytochelatins

Metal-treated cells were collected by filtration onto 1.2 μm membrane filters, re-suspended in 1.5 ml of 0.1 M HCl / 5 mM DTPA, then disrupted by sonication (Sonopuls Ultrasonic Homogenizer, Bandelin) for 3 min with a repeating duty cycle of 0.3 s, in an ice bath. The cellular homogenate was centrifuged (20000 g, 45 min) and the supernatant was used for the determination of thiols. Glutathione and phytochelatins were separated and quantified by high performance liquid chromatography (HPLC) after derivatization with the fluorescent tag monobromobimane (mBrB), by following the procedure reported elsewhere (Morelli and Scarano, 2001). Briefly, 400 μL of the sample were added to 200 μL of buffer (400 mM HEPPS (4-(2-hydroxyethyl)-piperazine-1-propane-sulfonic acid) / 5 mM DTPA, pH 9) and to 20 μL of 10 mM TCEP (Tris (2-carboxyethyl) phosphine) in order to reduce oxidized thiol groups. After 15 min of incubation, two successive reactions in the dark at 45°C for 15 min were carried out, following the addition of 40 mL of 10 mM mBrB and of 40 mL of 100 mM cysteine, respectively. Finally, 40 mL of 1 M methanesulfonic acid were added to stop the reaction. Analyses were performed on an HPLC system consisting of two Shimadzu LC-10AD pumps, a Rheodyne 7725 injection valve equipped with a 100 ml loop, a fluorescence

detector (RF–10AXL, Shimadzu) set at 380 nm excitation wavelength and 470 nm emission wavelength, and an Alltech Alltima (5 mm, 250 mm, 4.6 mm) C-18 reverse-phase column. An acetonitrile gradient in 0.1% trifluoroacetic acid (from 10% to 12% for 15 min and from 12% to 28% for a further 40 min) was used at a flow rate of 1 mL min^{-1}. Standard PC from *Silene vulgaris* (Friederich et al., 1998) were kindly provided by Prof. M.H. Zenk, Munich University (Germany), and were used to check the retention time of phytochelatin oligomers. PC quantification was obtained from the relationship peak area vs. concentration of reduced glutathione (GSH) standard solutions. The total cellular PC concentration was expressed as the sum of the γ-Glu-Cys units quantified in each chromatographic peak of phytochelatins.

3.10. Measurement of metal concentrations

Determination of heavy metals (Cd, Pb, Cu and Zn) in natural seawater samples and in the elutriates of marine sediments was carried out by using the electrochemical technique "Differential Pulse Anodic Stripping Voltammetry" (DPASV).

Voltammetric measurements were carried out by a Metrohm Model 646 VA processor in conjunction with a 647 VA Stand, equipped with a Metrohm multi-mode electrode (MME) used in the hanging mercury drop mode (HMDE). The instrumental settings were: scan rate 12 mV s^{-1}, pulse duration 40 ms, pulse amplitude 50 mV, pulse repetition time 0.5 s, deposition time 300 s; deposition potential (Edep) -1.2 V. The voltammetric sensitivities, obtained in seawater at pH = 2 spiked with increasing concentrations of $CdCl_2$ or $Cu(NO_3)_2$ or $Pb(NO_3)_2$ standard solutions, were 3.7×10^{-3}, 1.3×10^{-3} and 4.1×10^{-3} $A \times M^{-1} \times s^{-1}$ for Cd, Cu and Pb, respectively.

Determinations of heavy metals in seawater were performed at pH = 8.2 to measure the electrochemically labile fraction of the metal or at pH = 2 to measure the concentration of the total dissolved metal.

3.11. Dissolved Organic Carbon (DOC) measurement

DOC was measured using a Shimadzu 5000 TOC Analyzer. Ten mL samples were acidified with 50 µL of 50% H_3PO_4 and purged for 10 min with ultra pure oxygen to remove inorganic carbon ahead of high-temperature catalytic oxidation. One hundred µL of the experimental samples were injected into the furnace, after rinsing four times with aliquots of the sample (Sharp et al., 1993). Measurements were performed in triplicate with a fixed instrumental variance <2%. A DOC certified seawater standard (nominal value: 44-45 µM), provided by the Bermuda Biological Station (BBS, USA) was used to verify the accuracy of the measurements.

3.12. Determination of Dissolved Gaseous Mercury production

The Dissolved Gaseous Mercury (DGM) production was measured in cultures of *T. weissflogii* exposed to 5 nM mercury, at a constant temperature (21 °C), using the experimental apparatus described in the next paragraph, after removing the DGM initially present in the sample.

Measurements of DGM production were performed under light and dark conditions, both in the cultures *in toto* and in the culture media after removal of the cells by filtration, in order to discriminate between the biotic contribution to the mercury volatilization and that of the cellular exudates, well known for their photo-sensitising role in marine photochemical reactions (Costa and Liss, 1999; Lanzillotta et al., 2004). The cultures were filtered under sterile conditions using a 1.2 mm pore-size cellulose-nitrate filter (Sartorius 11403–47).

DGM production was also measured in the same cultures of *T. weissflogii* killed with formaldehyde, in order to eliminate any biotic contribution.

A 50 mL volume of the samples were transferred into 100 mL glass Pyrex purging bottle, showing optical properties elsewhere described (Lanzillotta and Ferrara, 2001), and a good transmittance (85%) for wavelengths > 350 nm.

Before each experiment to measure DGM production, samples were bubbled for 2 hours in dark conditions in order to reduce the significant DGM production resulting from the photo-activation of the organic matter released by the cells (Lanzillotta et al., 2004). In fact the intermediates formed during the growth-light could be responsible for Hg^{2+} reduction processes in the dark independent from the direct contribution of the cells.

Consecutive cycles of DGM determinations were performed in order to determine the extraction efficiency and the blank value. During the first extraction more than 95% of the DGM was extracted, in agreement with the mass balance over the impinger with phase equilibrium of Hg^0 between water and gas reported by Gardfeldt et al. (2002). Therefore two consecutive extractions of the DGM were always performed before determining the DGM production.

DGM production in darkness was obtained incubating the sample contained in the purging bottle for 20 min in the dark; the produced DGM was extracted under dark conditions. The DGM production under light conditions was recorded following the exposure of the purging bottle, containing the sample, for 20 min to the same fluorescent light used for culture growth (100 μmol photons $\times$ m^{-2} $\times$ s^{-1}).

Preliminary tests were performed to verify the period of incubation within which the DGM production was linear in time. An incubation time of 20 minutes was selected to obtain a meaningful DGM amount and to be within the linear time range of DGM production.

The DGM determinations were replicated 3 times.

3.12.1. Experimental apparatus for DGM determination

Measurement of DGM production was accomplished using the experimental apparatus shown in Fig. 3.3.

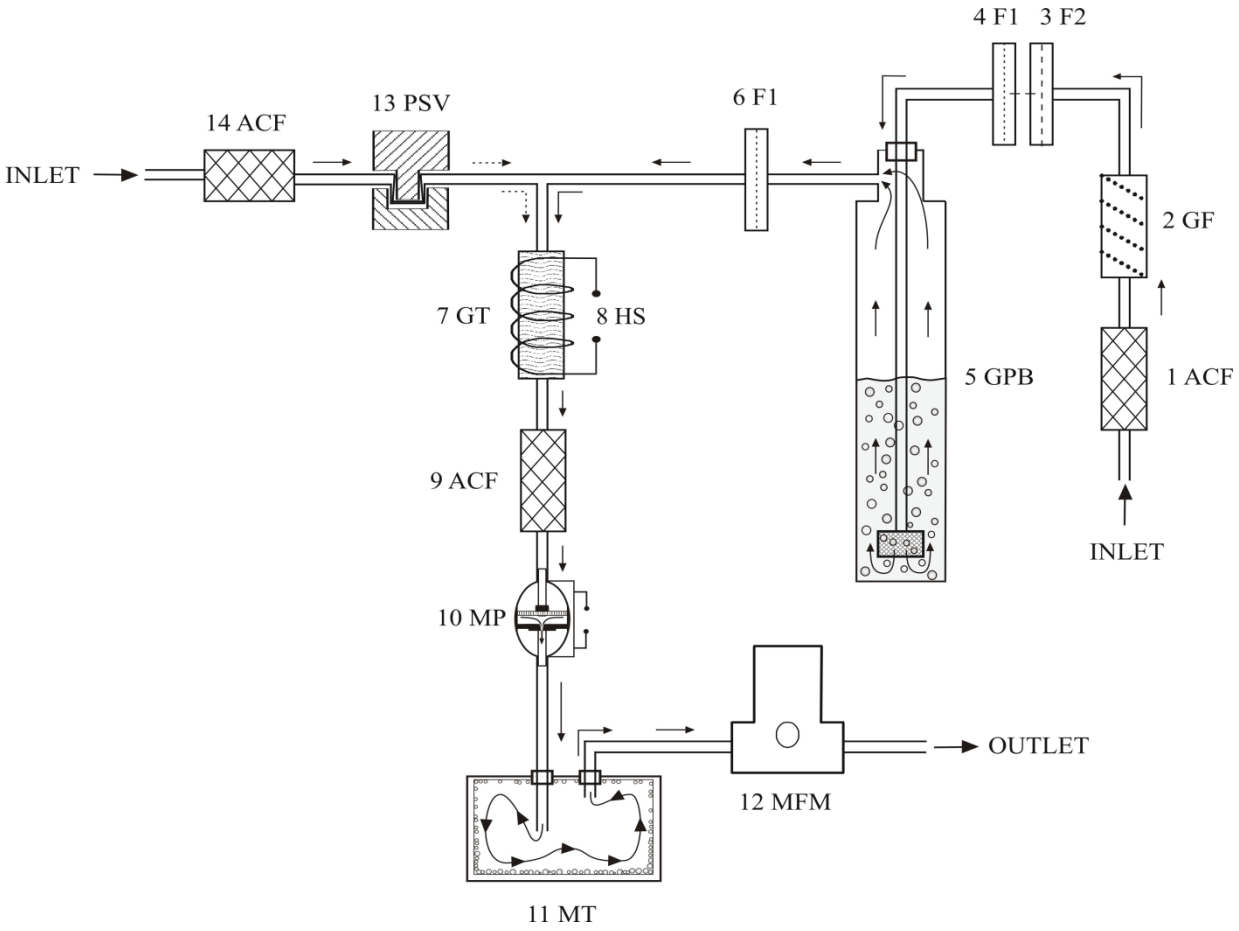

Fig. 3.3. Experimental apparatus for DGM determination. ACF = Activated Carbon Filter; PSV = Pinch Solenoid Valve; F1 = 0.20 μm Filter; F2 = 0.45 μm Filter; GF = Gold Filter; GPB = Glass Pyrex Bottle; GT = Gold Trap; HS = Heating System; MP = Membrane Pump; MT = Moisture Trap; MFM = Mass Flow Meter; PSV = Pinch Solenoid Valve.

External air was bubbled through the incubated sample in the glass Pyrex purging bottle (5GPB), at a flow rate of 0.7 L min^{-1} for 10 minutes, by using a membrane pump (10 MP). An iodated activated carbon filter (1 ACF) and a gold filter (2GF) provided mercury-free air to the impinger. The air flowed through two sterile filters of 0.45 μm (Sartorius 16555; 3 F2) and 0.20 μm (Sartorius SM16534; 4 F1) pore size, respectively. The DGM produced in the

sterilized purging bottle flowed through a sterile 0.20 μm (Sartorius SM16534; 6 F1) pore size filter and was preconcentrated on a gold trap (7 GT). This trap consisted of a small quartz tube (12.0 cm in length, 0.4 cm internal diameter), containing 1.0 g of gold wool mixed with quartz grains, kept in position by quartz wool.

A nickel-chromo wire, wrapped around the gold trap, served as a heating system (8 HS), ensuring a temperature of 50 °C during the DGM extraction process. This temperature was necessary to avoid moisture condensation in the trap itself, which would have caused a reduced efficiency of the mercury trapping.

A second activated carbon filter (9 ACF), inserted between the gold trap (7 GT) and the membrane pump (10 MP), protected the former against mercury contamination deriving from the membrane pump, the moisture trap (11 MT) and the mass flowmeter (12 MFM). The function of the moisture trap (11 MT) aimed at avoiding water condensation which could hamper the regular functioning of the mass flowmeter.

At the end of the extraction process, the air pressure inside the purging bottle was returned to atmospheric pressure by opening a solenoid pinch valve (13 SPV). The incoming external air was rendered mercury free by a third activated carbon filter (14 ACF).

All the experiments were performed at a constant temperature of 21 °C and the purging bottle, together with the teflon tubing, were pre-cleaned by acid washing every time before the experimental apparatus had to be involved in a new measurement cycle.

The mercury pre-concentrated on the gold trap was determined using a Tekran Cold Vapour Atomic Fluorescence Spectrometer 2500 described in the next paragraph. The trap was heated to 500 °C using the same heating element used to avoid moisture condensation; the released mercury was transported to the fluorescence cell using pure argon as a carrier gas.

3.12.2. The atomic fluorescence spectrophotometer

The elemental mercury collected on the gold trap, was determined after thermal desorption by using the fluorescence spectrophotometer Tekran 2500 (Fig. 3.4).

An argon flow rate of 0.03 L/min, controlled by an electronic flow control system, allows the desorbed mercury to be carried to the quartz fluorescent cell of the instrument. The use of argon gas is necessary to avoid the "quenching" of the mercury fluorescence. In Fig. 3.4 a transversal section of the fluorimetric detector is reported. A block made of aluminium contains the fluorescence cell, the excitation lamp (which emits a light beam at a wavelength of 253.7 nm), and the photo-multiplier placed at 90° to the excitation light beam. The block of aluminium permits the avoidance of sudden thermical excursions of the system. The detection limit is 0.1 pg of mercury.

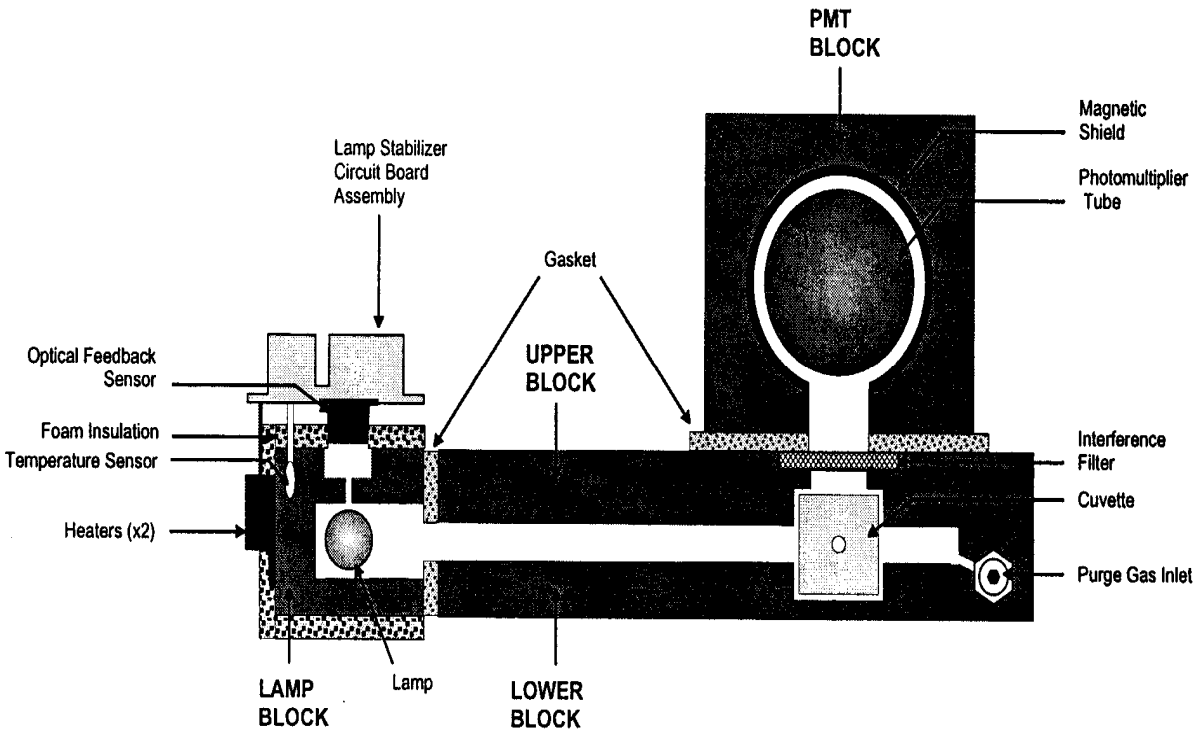

Fig. 3.4. Transversal section of the fluorimetric detector Tekran 2500.

The spectrophotometer was calibrated by injecting the concentrating gold trap with known increasing quantities of mercury saturated air via a gas-tight micro-syringe (Hamilton, 25 µL syringe volume). The known quantities of mercury were taken from a mercury vapour generator, constituted of a vessel kept at a temperature of 8°C, containing a few drops of mercury. The mercury passes from the liquid phase to the vapour phase in the air inside the vessel, until the air of the generator becomes saturated with mercury vapour. The internal pressure of the generator is kept at the ambient pressure. The mercury concentration inside the generator is a function of its temperature. The calibration procedure yielded very high reproducibility (2%) throughout seven replicas performed.

3.13. Determination of total dissolved mercury

Total dissolved mercury concentration ($[Hg]_{diss}$), was determined in the culture medium either inoculated or not inoculated with *T. weissflogii* cells. In the former culture medium, cells were removed by filtration (1.2 µm membrane filters, Millipore) before the Hg measurement. A calculated aliquot of the sample was diluted with distilled water to a final volume of 25 mL. Mineralization was carried out by adding 100 µL of HNO_3 and exposing the sample to the irradiation of a medium-pressure mercury vapour UV lamp (90W) for 5 min in a thermostatated bath at 2 °C.

Mercury was measured using the Atomic Absorption Spectrometer (AAS) Gardis-IA, (described in the next paragraph) after adding 200 µL of the $SnCl_2$ solution to reduce the Hg^{2+} dissolved in the sample to Hg^0, which is the only chemical species of mercury that can be detected by the spectrophotometer.

The extracted volatile mercury compounds are trapped on a concentrating gold trap, similar to that described in paragraph 3.12.1. The gold trap is placed outside the mercury analyser and kept at a temperature of about 50°C. The concentrating gold trap is automatically connected

to the spectrophotometer by means of a pinch solenoid valve. Mercury is thermally desorbed (at 500°C) from the concentrating trap and re-collected on the analytical gold trap placed inside the spectrophotometer. A second thermal desorption process releases mercury from the analytical trap and allows its determination by AAS. Freshly acid-cleaned Teflon tubing is used to connect the glass bottle to the measuring device.

The value of the blank of the measuring device was 2-4 pg of mercury.

3.13.1. The atomic absorption spectrophotometer

The scheme of the mercury vapour analyser Gardis-IA is reported in Fig. 3.5.

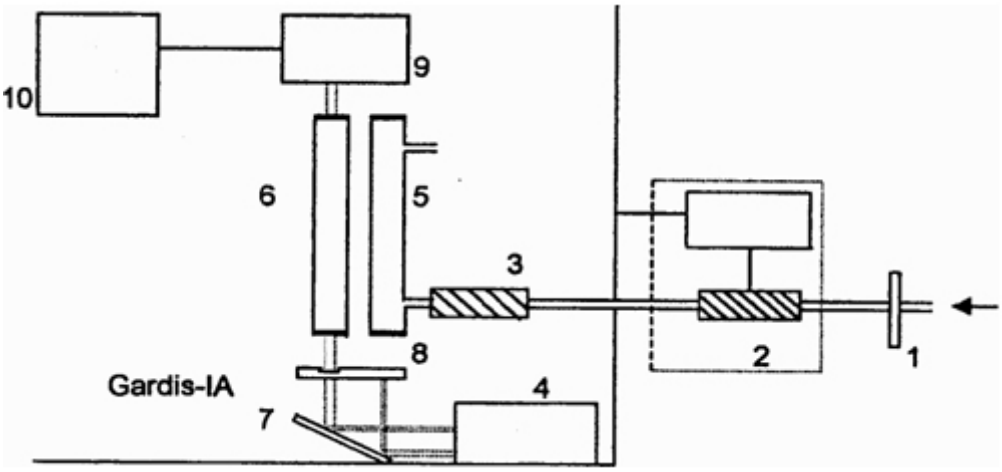

Fig. 3.5. Scheme of the atomic absorption spectrophotometer Gardis-IA.
1= teflon filter; 2= concentrating gold trap; 3= analytical gold trap; 4= mercury vapour UV lamp; 5= measuring absorption cell; 6= reference absorption cell; 7= mirror to direct the light beam toward the cells; 8= chopper to turn the light beam alternately toward the two cells; 9= photodetector; 10= amplifier.

The mercury thermally desorbed from the analytical gold trap passes the absorption cell of the spectrophotometer ("the measuring cell" which consists of a quartz tube 20 cm length, 3 mm internal diameter, 5 in Fig. 3.5). A small mirror (7 in Fig. 3.5) allows the light beam emitted

from the UV mercury vapour lamp (4 in Fig. 3.5) in the wavelength line of 253.7 nm to reach the absorption cell. If in the absorption cell atoms of elemental mercury are present, they absorb part of the light beam; in this way the light beam undergoes a decrease in its intensity in proportion to the quantity of Hg^0 atoms in the absorption cell. The light beam coming from the UV lamp passes alternately through the measuring absorption cell and through a reference absorption cell (6 in Fig. 3.5), identical to the measuring one. A photodetector (9 in Fig. 3.5) measures the difference in the intensity of the light beam coming from the two cells. In this way errors in measuring the quantity of mercury due to reflections inside the measuring cell and due to possible variations of the emission of the UV lamp are reduced.

The detection limit of the modified spectrophotometer was 0.5 pg of mercury.

For calibrating the system, the same methodology employed for calibrating the Tekran fluorescence spectrophotometer was used (paragraph 3.12.2).

3.14. Determination of cellular and intracellular concentration of mercury

Mercury-treated cells of *T. weissflogii* were collected by filtration onto 1.2 μm membrane filters (Millipore) and used for the determination of the total cellular mercury concentration ($[Hg]_{cell}$). In order to determine the $[Hg]_{intr}$, the harvested cells were incubated for 10 minutes with 1 mM EDTA in seawater to remove the metal adsorbed to the cell surface, then rinsed extensively with natural seawater. The cells of *T. weissflogii*, either rinsed with EDTA or not rinsed, were immediately placed in 1 mL of HNO_3 (0.14 M) in water and mixed with 1 mL of concentrated HNO_3 and H_2O_2 (2:1 v/v). The samples were digested at 45 °C for 16 h. This mineralization procedure was validated by using a Standard Reference Material (T6) "Fresh Water Plankton". The results of analysis on the Standard Reference Material was 0.173 ± 0.03 μg g^{-1} dw compared with that of 0.186 ± 0.04 μg g^{-1} dw reported by BCR.

Calculated aliquots of each mineralized sample were diluted with distilled water to a final volume of 25 mL and added with 200 μL of the $SnCl_2$ solution to reduce the Hg^{2+} dissolved in the sample solution to Hg^0, which is the only chemical species of mercury that can be detected by the spectrophotometer.

The mercury concentration has been then measured by AAS, using the same experimental methodology described for the determination of the dissolved mercury concentration (par. 3.13).

3.15. Chemicals

All reagents were analytical grade: diethylenetriaminepentacetic acid (DTPA), reduced glutathione (GSH), γ-glutamylcysteine (γ-Glu-Cys), cysteine and monobromobimane (mBrB) were from Fluka; 4-(2-hydroxyethyl)-piperazine-1-propane-sulfonic acid (HEPPS), tris(2-carboxyethyl)phosphine (TCEP), hydrogen peroxide (30% solution) and $HgCl_2$ were from Sigma; methanesulfonic acid (MSA) was from Merck; acetonitrile and trifluoroacetic acid (TFA), HPLC grade, were from Baker; HCl, HNO_3 Suprapur grade, formaldehyde (40%), $Cu(NO_3)_2$, $CdCl_2$, $Pb(NO_3)_2$, $Hg(NO_3)_2$ and $SnCl_2$ were from Carlo Erba. The solution of SnCl2 (0.4 M) in 1.2 M HCl was purged with charcoal-filtered air for 1 h in order to remove the mercury contamination.

All solutions were prepared weekly and stored in the dark at +4°C. Water was purified by a Milli-Q system (Millipore).

CHAPTER 4

RESULTS AND DISCUSSION

The experimental study developed during the PhD was carried out with different taxa of marine autotrophic protists exposed to environmentally relevant levels of dissolved Cd, Cu, Pb, Zn and Hg.

The research was developed following the lines reported below:

- laboratory experiments on the evaluation of the suitability to use the accumulation of phytochelatins (PC) as a biomarker of metal bioavailability in bioassays for the assessment of metal pollution in the diatom *P. tricornutum*.
- Application of the bioassay to natural seawater.
- Comparison of the PC response among five taxa of marine autotrophic protists.
- Evaluation of PC synthesis as biomarker of metal bioavailability in sediments.
- Investigation on the relative importance of two defence mechanisms acting in the diatom *Thalassiosira weissflogii* exposed to potentially toxic concentration of mercury: synthesis of phytochelatins and production of dissolved gaseous mercury.

4.1. Evaluation of phytochelatins in the diatom *P. tricornutum* as biomarker of metal exposure in marine waters

The research was initially focused on the evaluation of the feasibility of using phytochelatin induction as a biomarker of metal exposure in *P. tricornutum*. The choise to employ *P. tricornutum* for the early investigations on this topic was based on previous studies on the kinetics of PC synthesis in this diatom (Morelli and Scarano, 2001; 2004). *P. tricornutum* has been shown to be able to synthesize PC in response to the most common metals (Rijstenbil and Wijnholds, 1996; Morelli and Scarano, 2001; Ahner et al., 2002; Morelli and Scarano,

2004), and its use in marine bioassays is widely diffused because of its easy cultivation and metal tolerance (Horvatić and Peršić, 2007).

4.1.1. Exposure experiment of P. tricornutum in EDTA-buffered artificial seawater

A first set of experiments was carried out in EDTA-buffered artificial seawater, with the aim to evaluate if the PC response in *P. tricornutum* could be related to the free metal ion concentration in seawater.

Metal exposure experiments were carried out by incubating 2×10^8 cells of *P. tricornutum* for 1 h in 200 ml of Aquil medium containing EDTA and Cd or Pb or Cu at the desired free-metal ion concentration. By taking into account the detection limit of the PC-assay protocol (1 pmol GSH injected, peak area 120), the incubation of 2×10^8 cells allows a reliable detection of 0.5 amol γ-Glu-Cys units $cell^{-1}$, equivalent to 3.0 pmol GSH (peak area, 360).

PC chromatograms were obtained from reverse-phase HPLC determination of non-protein thiols on the cellular extracts. A sequential elution of the oligomers, from shorter to longer chains, occurred with increasing retention time.

The cellular total PC concentration assayed in the cells before incubation was 0.3 ± 0.2 amol γ-Glu-Cys units $cell^{-1}$ (n = 3).

Under our exposure conditions, cadmium began to induce PC at a calculated free-Cd (Cd^{2+}) concentration of 0.2 nM (pCd^{2+} 9.7) corresponding to a value of total inorganic cadmium (Cd') of 7.4 nM ($pCd' = pCd^{2+} + \log \alpha_{Cd}$, where $\alpha_{Cd} = Cd^{2+}/Cd' = 2.7 \times 10^{-2}$ is the coefficient calculated by MINEQL+ which corrects for inorganic side-reactions of cadmium in Aquil). Replicated 1-h exposures to Cd^{2+} in the range 0.1 - 50 nM ($10 > pCd^{2+} > 7.3$; $8.4 > pCd' > 5.7$) gave the dose-response curve pCd^{2+} vs total PC γ-Glu-Cys units reported in Fig. 4.1. The lowest concentration of free-Pb (Pb^{2+}) inducing a measurable cellular PC induction was 0.6

nM (pPb^{2+} 9.2) corresponding to a value of total inorganic lead (Pb') of 15.0 nM (α_{Pb} = $Pb^{2+}/Pb' = 4.0 \times 10^{-2}$). Figure reports the dose-response curve for Pb^{2+} in the range 0.1 - 16 nM ($10 > pPb^{2+} > 7.8$; $8.6 > pPb' > 6.4$). At $pPb^{2+} = pCd^{2+} = 8.0$ cellular PC were about 15 and 40 amol γ-Glu-Cys units $cell^{-1}$, respectively.

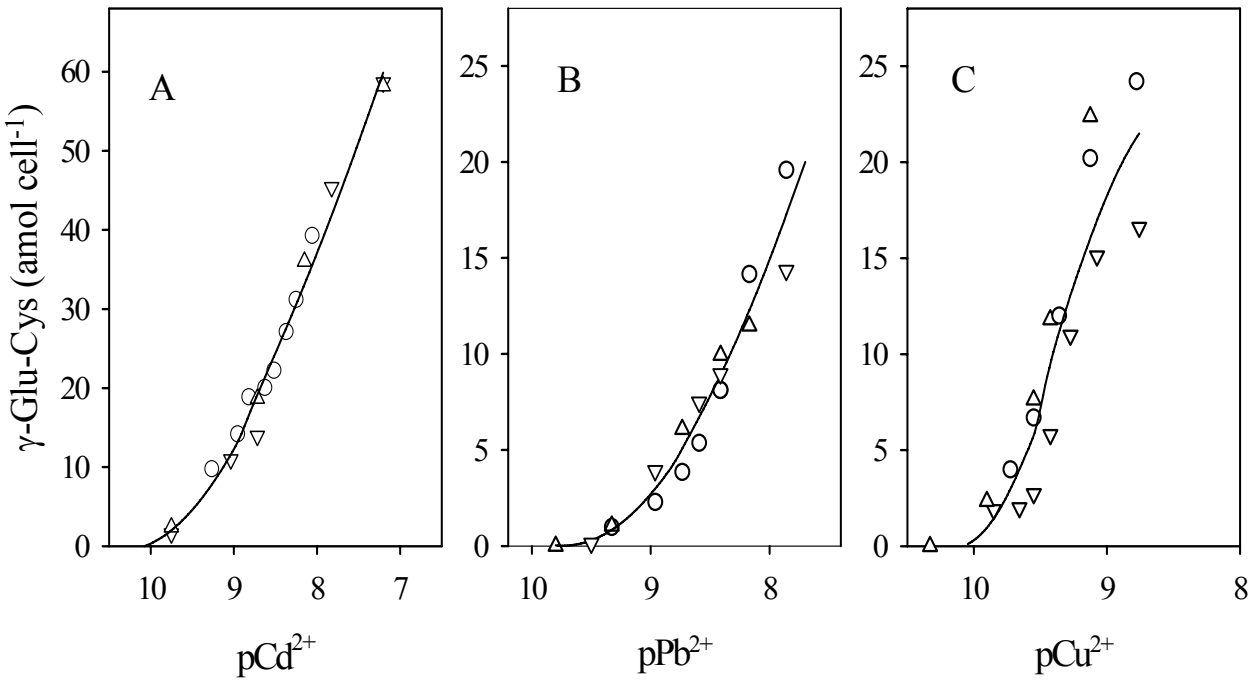

Fig. 4.1. Relationship between free metal ion concentration and phytochelatin synthesis in *P. tricornutum* after 1-h incubation in EDTA-buffered Aquil. Cell density, 1×10^6 cells mL^{-1}. Different symbols refer to 3 independent experiments. Phytochelatin concentration is expressed as the sum of the γ-Glu-Cys units in the individual oligomers.

Copper began to induce PC at a calculated free-Cu concentration of 0.2 nM (pCu^{2+} 9.7) corresponding to 3.2 nM of total inorganic copper (Cu') ($\alpha_{Cu} = Cu^{2+}/Cu' = 6.3 \times 10^{-2}$). Replicated 1-h exposures in the range 0.1 – 2.0 nM Cu^{2+} ($10 > pCu^{2+} > 8.7$; $8.8 > pCu' > 7.5$) gave the dose-response curve of Fig. 4.1. At $pCu^{2+} = pPb^{2+} = pCd^{2+} = 9.0$ cellular PC was about 18, 2.5 and 13 amol γ-Glu-Cys units $cell^{-1}$, respectively, showing that, at this low

concentration, Cu and Cd induced similar amounts of PC, whereas Pb was the weakest PC inductor.

On the basis of the relationships reported in Fig. 4.1, it can be concluded that in a medium at fixed free metal ion concentration, the cellular pool of phytochelatins in the diatom *P. tricornutum* increases as the concentration of free metal ions Cd, Pb and Cu increases in solution, behaving as a biomarker of exposure to the bioavailable fraction of these metals in solution. These results agree with earlier studies (Ahner et al., 1995; Ahner and Morel, 1995) carried out in a number of representative marine autotrophic microorganisms grown in steady-state conditions in EDTA-buffered Aquil. These studies showed that the PC production is an increasing function of the free metal ion in the medium, even if the degree of PC synthesis varies widely among the species. The present results at low concentration of fixed free metal ions suggest that the presence of PC in this diatom can represent an useful biological indicator for the assessment of metal pollution in marine waters.

4.1.2. Exposure experiment of P. tricornutum in natural seawater added with known amounts of heavy metals for evaluating the applicability of the bioassay to natural seawater

Application of the bioassay to natural seawater, added with known amounts of Cd, Pb or Cu, was tested by examining the dose response relationship between PC synthesis and metal concentration. Based on previous studies on the kinetics of PC synthesis in *P. tricornutum* (Morelli and Scarano, 2001; 2004), which showed an increasing PC accumulation within few hours from Cd, Pb or Cu addition, a 6 hour incubation time was used. The results (Fig. 4.2) showed linear dose-response relationships in the ranges $4 \leq [Cd_{lab}] \leq 100$ nM, $20 \leq [Pb_{lab}] \leq 200$ nM, $10 \leq [Cu_{lab}] \leq 200$ nM, indicating that cellular PC accumulation increases as a function of the labile metal concentration initially present in seawater. Since this operationally

defined metal fraction comprises both inorganic and electrochemically dissociable metal organic complexes (Achterberg and Braungardt, 1999), these results demonstrate the suitability of PC as a biomarker of exposure to the bioavailable forms of the metal dissolved in natural seawater, in agreement with the previous findings in media at fixed free metal ion concentration.

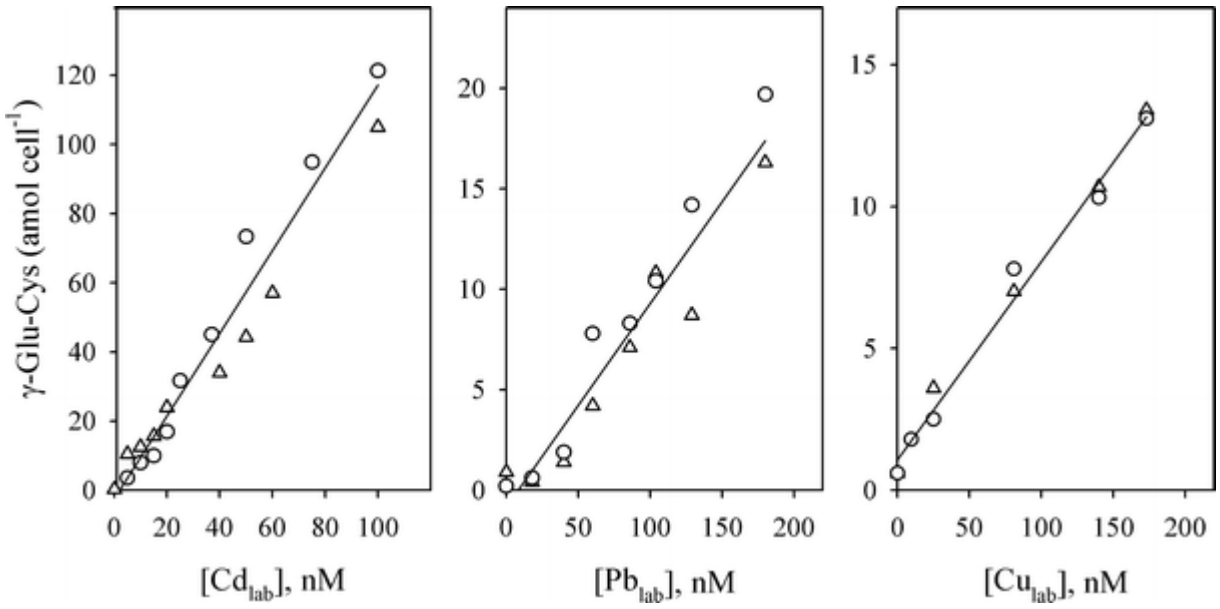

Fig. 4.2. Relationship between electrochemically labile metal concentration in seawater and phytochelatin synthesis in *P. tricornutum* after 6-h incubation in metal-enriched natural seawater. Cell density, 1×10^6 cells mL^{-1}. Different symbols refer to 2 independent experiments. Phytochelatin concentration is expressed as the sum of the γ-Glu-Cys units in the individual oligomers.

In natural seawater, the absence of a metal buffer hinders the relationship between PC and pMe^{2+} to be obtained, thus a comparison with the EDTA-buffered artificial seawater cannot be accomplished. In fact, cell-mediated processes, such as the adsorption on cell surfaces and/or the release of extracellular organic ligands able to form biologically inert complexes with the metal ions, can affect the concentration of the metal ion in solution.

4.1.3. Application of the bioassay to polluted seawater

As an application of the bioassay to polluted natural seawater, 6-h incubation tests were carried out on six different samples, collected along the coast in the province of Livorno, as shown in the map of Fig. 3.2.

The results showed that these waters were able to induce PC synthesis enhancing the cellular pool with concentrations ranging from 7.0 to 10.1 amol γ-Glu-Cys units cell^{-1} (Tab. 4.1).

Sample	Cd	Pb	Cu	Zn	PC [b]
Station 1	0.4 ± 0.1	0.9 ± 0.2	60 ± 11	80 ± 10	8.2 ± 1.5
Station 2	0.1 ± 0.1	1.6 ± 0.4	54 ± 7	123 ± 12	7.0 ± 0.3
Station 3	0.3 ± 0.2	0.9 ± 0.3	38 ± 5	61 ± 8	7.0 ± 0.6
Station 4	0.4 ± 0.3	0.8 ± 0.2	40 ± 6	88 ± 11	8.2 ± 1.7
Station 5	0.2 ± 0.1	1.5 ± 0.5	36 ± 4	72 ± 8	7.3 ± 1.4
Station 6	9.3 ± 0.3	1.4 ± 0.1	36 ± 7	102 ± 6	10.1 ± 0.4
EDTA-SS [c]	-	-	-	-	0.5 ± 0.4 [d]
Control seawater	0.1 ± 0.1	0.7 ± 0.1	7 ± 2	15 ± 2	0.3 ± 0.2

Table 4.1. Total dissolved metal concentrations (nM) in the sampling stations (map of Fig. 3.2) and phytochelatin concentration in cells of *P. tricornutum* after 6-h incubation in the seawater samples[a].

[a] Cell density, 4×10^5 cells ml^{-1}; [b] PC are expressed as γ-Glu-Cys units (amol cell^{-1}). The results refer to three independent experiments; [c] EDTA-treated (10μM) seawater samples; [d] n = 6; Metal concentrations are mean values ± S.D. of two to three measurements carried out at pH = 2 by ASV.

Incubation tests were then repeated on the same samples previously treated with EDTA (10 μM) and left to stand overnight. Incubations in such EDTA-treated waters produced no enhanced PC synthesis with respect to cells before incubation (0.5 ± 0.4 amol γ-Glu-Cys units

cell^{-1}). Since EDTA is a strong metal ion complexing agent, this result shows that the PC-inducing forms in these waters are metal ions in a bioavailable form, that are converted by EDTA in biologically inert complexes.

The PC synthesis induced by such samples must be considered a cumulative response to all metal ions able to induce PC in this diatom. Anodic Stripping Voltammetry (ASV) measurement of the more common PC-inducing metal ions were performed. ASV assays, at pH = 2, revealed the concentrations of total Zn, Cd, Pb, and Cu reported in Tab. 4.1.

Cd and Pb levels were similar to those assayed in the control seawater (Island of Capraia, Tyrrhenian Sea), except in station 6, in which Cd was one order of magnitude higher. In all the assayed samples, Zn and Cu were higher than in the control seawater. It has been reported (Morelli and Scarano, 2001; Kawakami et al., 2006a) that cells of *P. tricornutum* exposed to Zn at 1 μM did not exhibit PC synthesis in short-term experiments; hence the hypothesis that copper is the main accountable for the observed response could be made.

According to Wei et al. (2003) and Le Faucheur et al. (2005a), PC production can be not only related to the exposure to a single metal, but is the result of antagonistic and synergistic effects of multiple metals.

Kawakami et al. (2006b) have reported that Zn did not affect the amount of PC induced in *P. tricornutum* cells by Cu, whereas it had an antagonistic effect on the induction of PC by Cd. Hence, the inability of Zn to affect the PC response to Cu in *P. tricornutum* confirms the hypothesis that the induction of PC in this diatom incubated in our seawater samples was triggered by copper, although a contribution of Cd in station 6, which exhibited the highest PC response, cannot be excluded. In coastal seawater, copper is almost entirely complexed by organic ligands (Buck et al., 2007), but it is possible that in areas impacted by anthropogenic and industrial inputs the copper concentration exceeds that of the organic ligands, resulting in high levels of free Cu^{2+}. In our metal-polluted seawater samples the exact speciation of the

metal ions cannot be ascertained, nevertheless the ability of these waters to induce PC means that a fraction of the total dissolved metals (mainly copper) was in a bioavailable form, potentially toxic, at a concentration sufficient to activate a cellular detoxification system. Although many studies have reported that several planktonic marine and freshwater autotrophic microorganisms are able to synthesize PC in response to toxic heavy metals (Kawakami et al., 2006a) only few attempts have been made to use phytochelatins as biomarkers in a metal - specific bioassay (Wei et al., 2003; Le Faucheur et al, 2005a). The present results show that the PC response of the marine diatom *P. tricornutum* could represent a useful biomarker for the assessment of marine and freshwater toxicity.

4.2. Comparison of PC response in different taxa of autotrophic protists

On the basis of the knowledge acquired on PC synthesis in the diatom *P. tricornutum*, exposure experiments were carried out in order to compare the PC response in five taxa of autotrophic protists and to identify the most suitable test organisms for the development of further bioassays. To achieve this purpose, besides *P. tricornutum*, cultures of *T. weissflogii*, *S. costatum, D. tertiolecta and E. huxleyi* were incubated for 5 hours in natural seawater enriched with known amounts of Cd, Pb or Cu. The exposed cells were harvested and assayed for PC. For purposes of comparison among protist species largely different in size, the PC cellular concentration was normalized to the cell volume. Metal concentrations were measured by Anodic Stripping Voltammetry (ASV) at pH = 8, after equilibritation (16 hours after metal spiking) and before cells addition in the metal enriched media. These determinations revealed the electrochemical labile concentrations of the metals, which were assumed to correspond to the inorganic fraction of the metals (Me'), in agreement with Achterberg and Braungardt (1999).

Results obtained on the PC synthesis as a function of the inorganic fraction of metal concentrations are reported in Fig. 4.3. Figure clearly shows that all the microorganisms tested are capable to synthesize PC in response to metal exposure and that the PC response is species-specific.

As regards the PC synthesis in response to Cd exposure, the results show that in the three diatoms, PC were detected in cells even from the lower metal concentration ([Cd'] = 5 nM); thereafter PC begun to accumulate as the concentration of Cd increased in solution until reaching an intracellular concentration nearly to 600 μM at [Cd'] = 100 nM. On the contrary, in the case of *D. tertiolecta and E. huxleyi*, only low levels of PC were detected in the cells exposed to Cd.

As regards Cu exposure, results show that *P. tricornutum* and *T. weissflogii* were the most sensible to this metal among the five taxa of protists, being capable to synthesize a total cellular PC of 100 μM at the highest Cu concentration; differently, *Skeletonema costatum, Dunaliella tertiolecta and Emiliania huxleyi* showed low cellular PC contents for the entire range of Cu concentrations assayed.

As regards Pb exposure, results show that this metal induced PC synthesis enhancing the cellular pool with concentrations that in *S. costatum* were two times higher than in *P. tricornutum* and in *T. weissflogii*. The responses in PC production exhibited by *E. huxleyi* and *D. tertiolecta* were low as in the case of Cd and Cu exposure.

As a general rule, results point out a higher sensitivity to heavy metal stress of the three diatoms with respect to the coccolitophorid and the green algae. Moreover our results confirm that Cd represents the strongest inducer of PC synthesis, in agreement with the literature (Kawakami et al., 2006a; Ahner and Morel, 1995; Ahner et al., 2002).

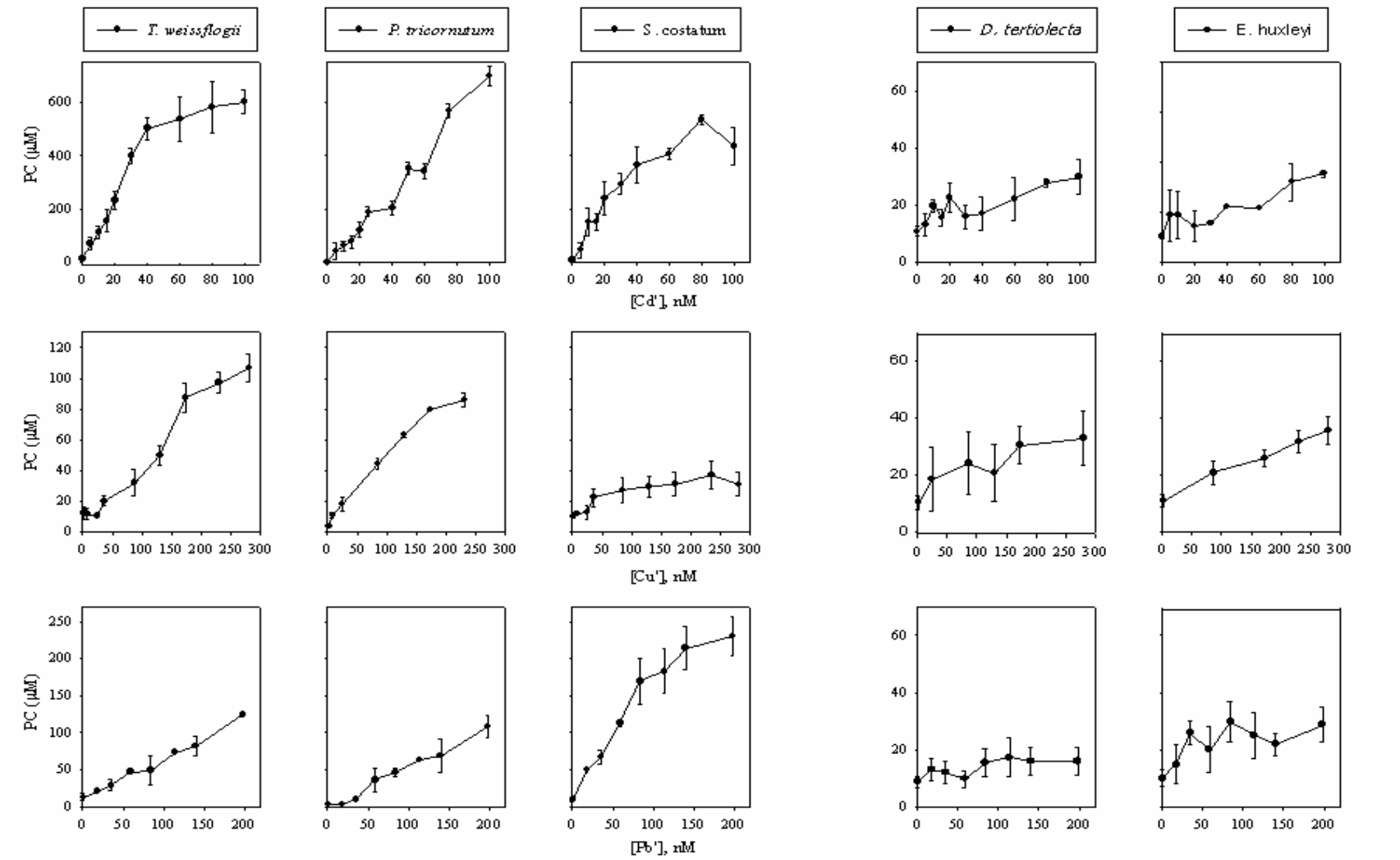

Fig. 4.3. Comparison of PC response among 5 different taxa of autotrophic protists incubated for 5 hours in natural seawater enriched with known amounts of Cd, Pb or Cu. Error bars correspond to the standard deviation (n = 2).

The low PC synthesis of *D. tertiolecta* and *E. huxleyi* in response to the different heavy metals tested could be explained by the occurrence of other detoxification mechanisms, which could be involved in these microorganisms against metal stress. Among these, Gonzales-Davila et. al (1995) and Leal et al. (1999) observed the production of cellular exudates capable to bind the metal in solution in *D. tertiolecta* and *E. huxleyi* exposed to Cu.

4.3. Evaluation of PC synthesis in *P. tricornutum*, *T. weissflogii* and *S. costatum* as biomarker of metal bioavailability in sediments

On the basis of the results previously described, the three marine diatoms have been used to develop a new bioassay involving the presence of phytochelatins as response to metal bioavailability in re-suspensions of metal-polluted marine sediments.

For this purpose, laboratory cultures of *P. tricornutum*, *T. weissflogii* and *S. costatum* were exposed to elutriates of sediments collected at two sites (named A and B) in a polluted coastal area in the province of Pisa (Tuscany, Italy) (see map of Fig. 3.1).

Chemical characterization of the elutriates in terms of concentration of total dissolved metal concentration, pH and dissolved organic carbon (DOC) is reported in Tab. 4.2.

Samples	[Cd], nM	[Pb], nM	[Cu], nM	pH	DOC (µg L^{-1})
Filling Channel of the Arno River (Elutriate A)	3.5±1.2	1.2±0.2	41.0±2.9	7.8	527
Marina di Pisa (Elutriate B)	16.1±1.1	0.4±0.1	11.0±3.4	8.2	267
Control seawater	0.1±0.1	0.7±0.1	5.2±2.5	8.2	71

Tab. 4.2. Total dissolved metal concentrations, dissolved organic carbon (DOC) and pH in the elutriates of sediments collected at sites A and B (map of Fig. 3.1) and in the control seawater used for elutriate preparation. Metal concentrations are mean values ± S.D. of three measurements carried out at pH = 2 by ASV.

The results show that the sediment re-suspension increased the total metal concentration in the control seawater. The elutriate of sediment A exhibited a higher copper concentration than the elutriate of sediment B, which in turn was richer in Cd. The release of lead was negligible in both elutriates. DOC concentrations revealed a higher release of organic compounds from the sediment A compared to the sediment B.

Short- and long-term incubations were carried out in both the elutriates.

4.3.1. Short-term incubations

Short-term exposure experiments were carried out by using not-growing cells of *P. tricornutum*, *T. weissflogii* and *S. costatum*, coming from precultures at the end of the logarithmic growth phase. The diatoms were incubated for 5 h in the elutriates of sediments A and B, diluted with natural seawater to concentrations of 0 (control), 25, 50, 75 and 100 % , and assayed for PC production. The results (Fig 4.4) show that the three species of diatoms responded to the exposure to both elutriates by synthesizing PC in increasing amounts with respect to the percentage of the elutriate present in solution.

Since PC are a cellular response to increased intracellular free metal ions, this finding indicates that both sediments released bioavailable metal species not present in the control seawater, which would be accumulated by the phytoplanktonic protists so as to induce PC synthesis. Thus, the presence of PC in these microorganisms can be considered a suitable biomarker of exposure to the bioavailable metal fraction released by the contaminated sediments. For the elutriate A, PC content in *P. tricornutum*, *T. weissflogii* and *S. costatum* significantly increased (t-test, $p < 0.05$) between the control and the 25% concentration elutriate, thereafter it rose till to reach the values of 50 ± 6, 31 ± 4 and 22 ± 5 μM, respectively, for the 100% elutriate.

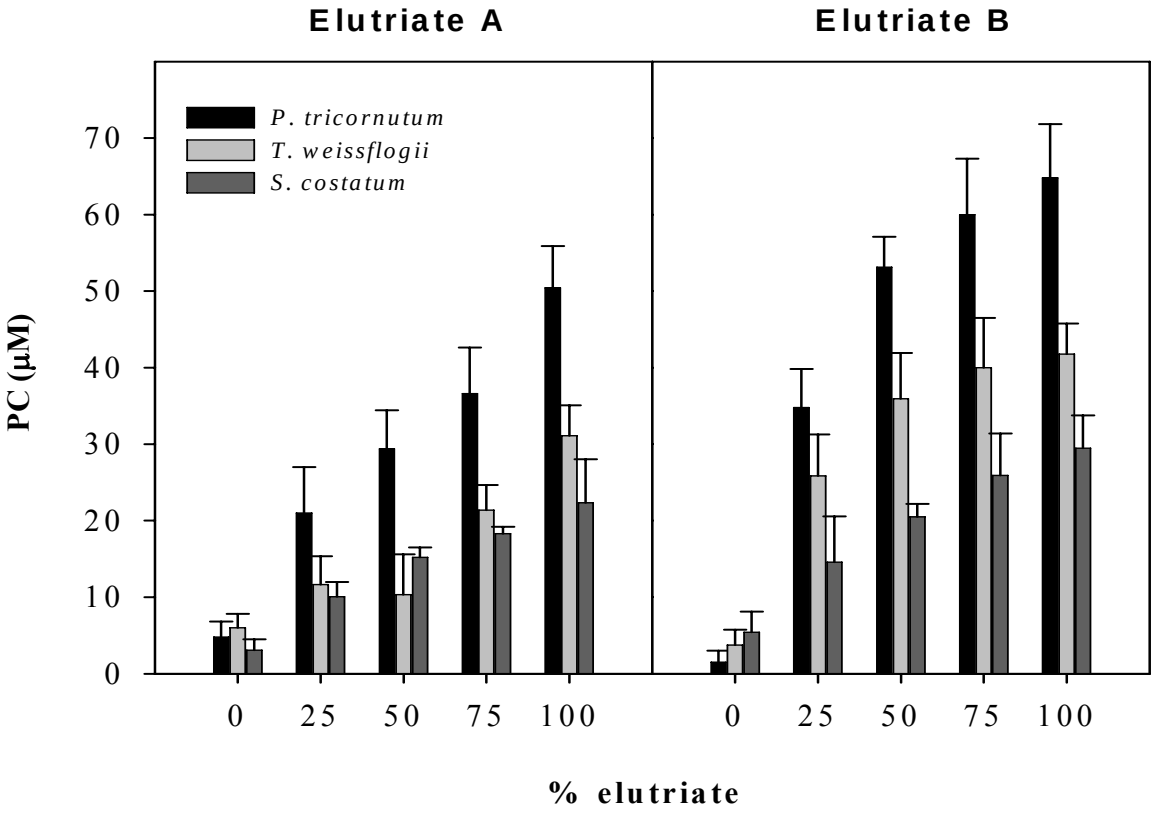

Fig. 4.4. Phytochelatin concentration in cells of *P. tricornutum*, *T. weissflogii* and *S. costatum* exposed for 5 hours to different concentrations of the elutriate A and of the elutriate B. Error bars correspond to the standard deviation (n = 3).

A similar behavior was observed for the elutriate B, in which *P. tricornutum*, *T. weissflogii* and *S. costatum* produced a maximum amount of PC of 65 ± 7, 42 ± 4 and 30 ± 4 µM, respectively, in cells exposed to the 100% elutriate. These results suggest that the capability of the diatoms to synthesize PC, under these exposure conditions, was in the order *P. tricornutum* > *T. weissflogii* > *S. costatum*. Elutriate B resulted more effective to induce PC than the elutriate A, presumedly for the higher content in Cd, which is considered to be a stronger PC inductor (Ahner and Morel, 1995; Rijstenbil and Wijnholds, 1996). Moreover, the higher content of organic compounds in the elutriate A (see the DOC value in table 4.2) could account for a lower metal bioavailability by means of complexation reactions.

4.3.2. Long-term incubations

Long-term exposure experiments were carried out in cultures of *P. tricornutum*, *T. weissflogii* and *S. costatum* inoculated in the elutriates of sediments A and B, from 0% to 100% concentration, and let grow for 6 days. This approach allowed to evaluate the effect of the elutriate exposure both on the PC response in exponentially growing cells and on the growth rate of the protist cultures. As shown in Fig. 4.5, PC assays exhibited the presence of PC in all species of phytoplankton examined, with a general increase of their concentration in function of the concentration of the elutriate. The pattern of PC in these exponentially growing cells, in agreement with that observed in not-growing cells (5 hours exposure), confirms the direct relationship between PC and elutriate concentration, supporting the suitability of the use of PC as a metal exposure biomarker.

However, large differences were observed among the species. *P. tricornutum* responded to the elutriate exposure by synthesizing very low amounts of PC, lower than in not-growing cells incubated for 5 hours in the same media, showing a scarce sensitivity of this diatom in the exponential growth phase to the metal exposure. On the contrary, the PC responses in *T. weissflogii* and *S. costatum* in the long-term incubations were higher than that in the short-term incubations, as expected for a longer incubation time. Compared to the other species, *T. weissflogii* responded to the elutriate exposures by synthesizing the highest amounts of PC, reaching intracellular PC concentration of 498 ± 43 μM in cells exposed to the 100% elutriate A (with respect to 40 ± 6 μM PC in *S. costatum* and 14±4 μM PC in *P. tricornutum*) and of 2380 ± 200 μM in cells exposed to the 100% elutriate B (with respect to 393± 53 μM PC in *S. costatum* and 11 ± 6 μM PC in *P. tricornutum*).

The capability of the diatoms during their exponentially growth phase to synthesize PC was in the order: *T. weissflogii* > *S. costatum* > *P. tricornutum*.

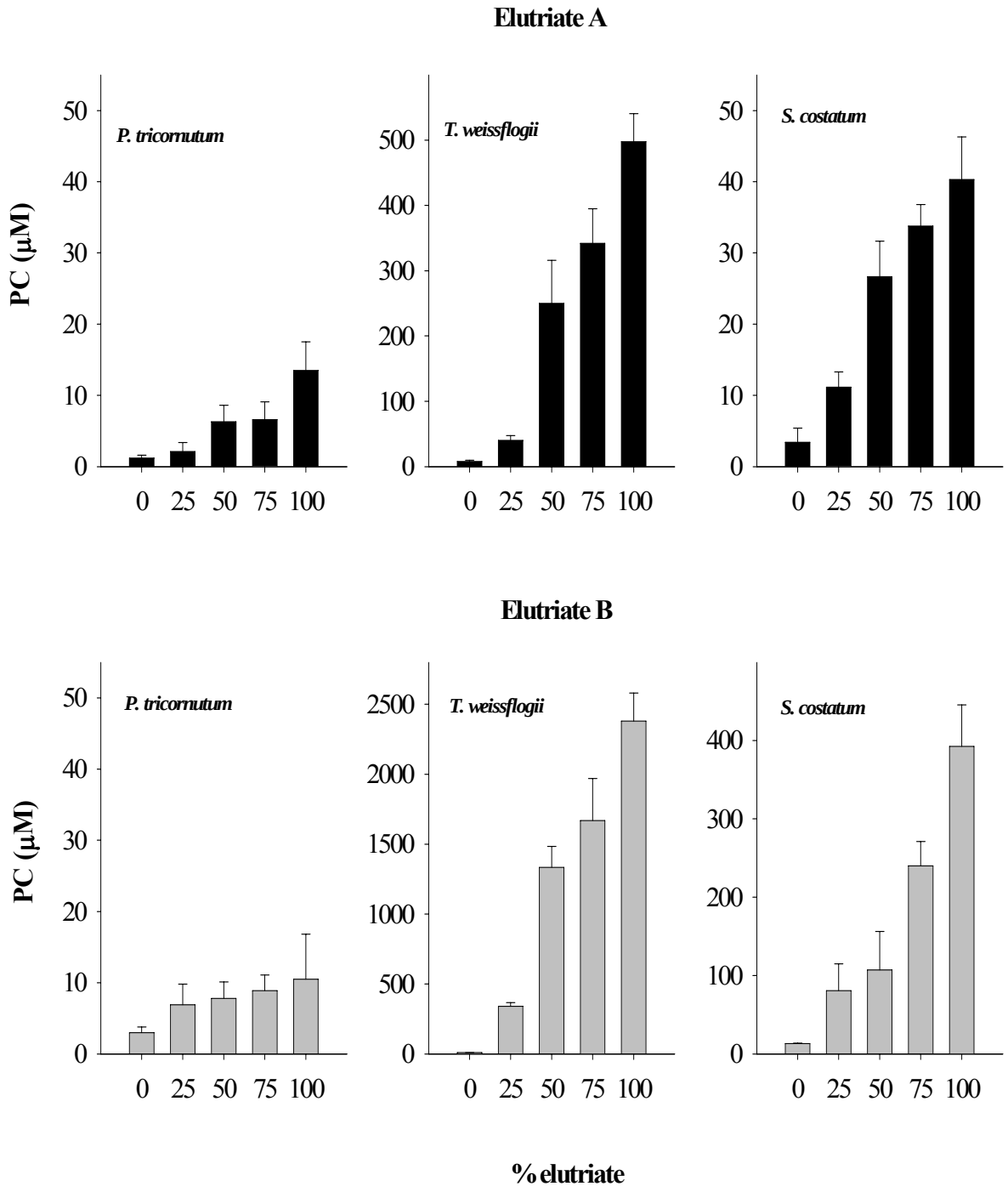

Fig. 4.5. Phytochelatin concentration in cells of *P. tricornutum*, *T. weissflogii* and *S. costatum* grown for 6 days in different concentrations of the elutriates A and B. Error bars correspond to the standard deviation (n = 3).

Compared to the other diatoms, *P. tricornutum* is smaller in size (see Tab. 3.1) and grows faster, hence a higher dilution of the intracellular metal concentration could occur in this diatom during the exponential growth phase, thereby limiting the metal accumulation and hence PC synthesis. In agreement, low levels of PC during the exponential growth phase were earlier observed in *P. tricornutum* (Morelli et al., 2002) grown in the presence of Cd (1 μM). Cellular Cd assays showed that the scarce ability of the cells to synthesize PC during the exponential phase was concomitant with a low Cd uptake, suggesting that in this diatom the process of cell division can be accompanied by a mechanism of limitation of metal accumulation and, hence, of PC synthesis.

According to the short-term incubations, the elutriate B stimulated PC synthesis at a greater extent than elutriate A, as evidenced in *T. weissflogii* and *S. costatum* cells. This difference was enhanced in the long-term incubations compared to the short-term ones, thus indicating that the former approach is more sensitive than the latter one.

Both elutriates A and B had not any inhibitory effect on the growth of three diatoms examined, as evidenced from the growth rates reported in Tab. 4.3, rather a slight, but not significant (t-test, $p > 0.05$) stimulation of growth was sometimes observed in *P. tricornutum* and *S. costatum* cultures. From these data it should be inferred that both elutriates were not toxic for any of the microorganisms, nevertheless, the fact that the elutriate exposure induces PC synthesis in the same microorganisms means that a fraction of the heavy metals released by sediments is in a bioavailable form, potentially toxic, since it is able to activate a mechanism of detoxification.

The finding that the PC response occurs still before that growth is affected makes PC an early warning marker of exposure to heavy metals, useful to predict their potential toxicity.

The present data are supported by earlier studies (Ahner et al., 1994; Ahner and Morel, 1995) showing that, in metal-buffered artificial seawater, PC accumulation occurred at low free

metal ion concentrations, while the growth rate of the phytoplanktonic cultures did not change compared to a control culture.

	P. tricornutum **growth rate**	***T. weissflogii*** **growth rate**	***S. costatum*** **growth rate**
Elutriate A			
0%	1.67±0.05	1.06±0.04	1.38±0.02
25%	1.67±0.15	1.11±0.02	1.51±0.27
50%	1.64±0.29	1.07±0.01	1.34±0.27
75%	1.63±0.18	1.07±0.04	1.53±0.12
100%	1.72±0.18	1.05±0.03	1.66±0.21
Elutriate B			
0%	1.69±0.02	1.08±0.07	1.37±0.02
25%	1.87±0.13	1.08±0.02	1.43±0.01
50%	1.92±0.19	1.05±0.02	1.40±0.02
75%	1.86±0.17	1.08±0.20	1.42±0.11
100%	1.75±0.04	1.08±0.11	1.41±0.09

Tab. 4.3. Growth rate (μ, dublings day^{-1}) of *P. tricornutum*, *T. weissflogii* and *S. costatum* in the control and in the four concentration of elutriates A and B, calculated during the exponential growth phase.

4.3.3. Effect of cellular density on PC production

Additional long-term experiments (6 days) were carried out in order to examine the effect of cellular density in PC production. Cultures of *T. weissflogii* were inoculated at increasing cellular density in the elutriate A (50% concentration) and assayed for their PC content. The results show that PC accumulation in this diatom increased at decreasing initial cellular

density (Fig. 4.6). As can be seen from the figure, PC cellular concentration increased around three times when initial cell density decreased by one order of magnitude (from 1×10^3 to 1×10^2 cells mL^{-1}).

Our findings well agree with the results of Moreno-Garrido et al. (2000) which, performing growth inhibition tests on a variety of marine microalgal species inoculated in metal-enriched media, found an inverse relationship between initial cellular density and sensitivity of the test; these authors suggested that under the same metal concentration in the media, cells at lower cellular densities accumulate a higher amount of metal. Thus, the enhancement of PC synthesis occurring in *T. weissflogii* at lower cellular density can be exploited to improve the sensitivity of the bioassay. Analogous experiments performed with cultures of *P. tricornutum* grown for 6 days in the elutriate A (100% concentration) showed that the PC accumulation was very low and was only weakly affected by the initial cellular density (Fig. 4.6).

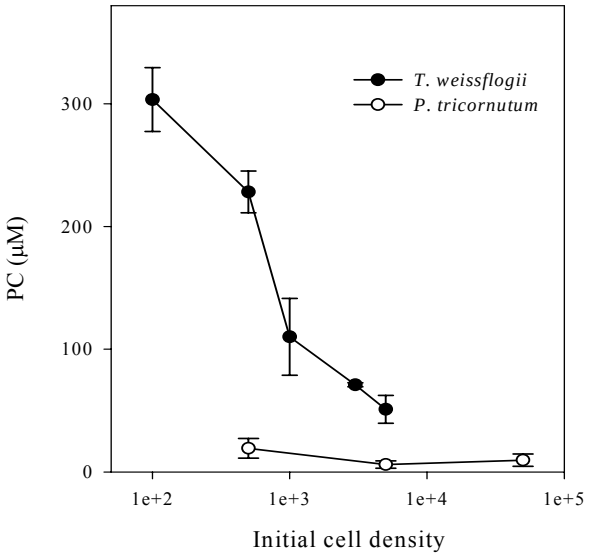

Fig. 4.6. Effect of the initial cell density (cells mL^{-1}) on PC production in cultures of *T. weissflogii* (•) and of *P. tricornutum* (o) grown for 6 days in the 50% and 100% elutriate A, respectively. The experiment was carried out in duplicate, and bars correspond to the standard deviation.

This finding confirms the scarce ability of exponentially growing cultures of *P. tricornutum* to produce PC, as observed in the long-term exposures in the elutriates A and B.

Taken togheter results show that the initial cellular density in incubations carried out during the exponential growth phase can be a critical factor in PC response, therefore, for evaluating and comparing the quality of different sediment samples, the use of standardized exposure conditions is recommended.

4.3.4. Application of the bioassay to a polluted area

Among the phytoplanktonic species assayed, *T. weissflogii* in the exponential growth phase resulted the most sensitive to the elutriate exposure, so this diatom was chosen for an application study in a coastal area contaminated by heavy metals.

The PC-induction test (6 days exposure) was carried out on the elutriates from twelve marine sediments collected in a coastal area at north of the mouth of the Arno river (map showed in Fig. 3.1). Total concentrations of the more common PC-inducing metal ions in the elutriates from such sediments were measured by Anodic Stripping Voltammetry (ASV) and are reported in Tab. 4.4.

Data show that the sediments released mainly Cd and, at a lesser extent, Cu, whereas the contribution of Pb was very low.

Although a complete analysis of metals in these samples was not performed, the concentration of the measured metals can be considered as an indicator of the degree of contamination of these sediments.

It was not possible to distinguish which metal may be responsible for the PC response, but the PC induction by such elutriates must be considered a cumulative response to all metal ions able to induce PC in this diatom.

Station	[Cd]	[Pb]	[Cu]
1	27 ± 6	b.d.	13 ± 6
2	40 ± 4	b.d.	12 ± 1
3	37 ± 3	0.3 ± 0.1	16 ± 2
4	49 ± 5	0.3 ± 0.1	10 ± 2
5	40 ± 12	1 ± 0.5	12 ± 4
6	48 ± 5	0.5 ± 0.3	8 ± 1
7	47 ± 7	0.5 ± 0.2	7 ± 0.5
8	38 ± 8	1.5 ± 0.5	17 ± 5
9	167 ± 15	0.6 ± 0.2	53 ± 5
10	153 ± 12	0.3 ± 0.2	24 ± 6
11	59 ± 6	1 ± 0.2	11 ± 2
12	230 ± 26	0.6 ± 0.2	57 ± 7

Tab. 4.4. Total dissolved metal concentration (nM) in the elutriates obtained from the twelve sediments collected in a contaminated coastal area (map showed in Fig. 3.1). Standard deviation is referred to duplicate measurements carried out by ASV at pH = 2; b.d. = below detection.

As shown in Fig. 4.7, cellular PC assays carried out on the elutriates showed that all samples were able to induce PC synthesis in *T. weissflogii* cells, exhibiting an increasing PC concentration as the total dissolved metal concentration increased.

The occurring of a positive relationship between cellular PC and the degree of contamination, confirms the suitability of the use of PC as a biological indicator of metal biovailability and, hence, of potential toxicity of heavy metals in the elutriates of marine sediments.

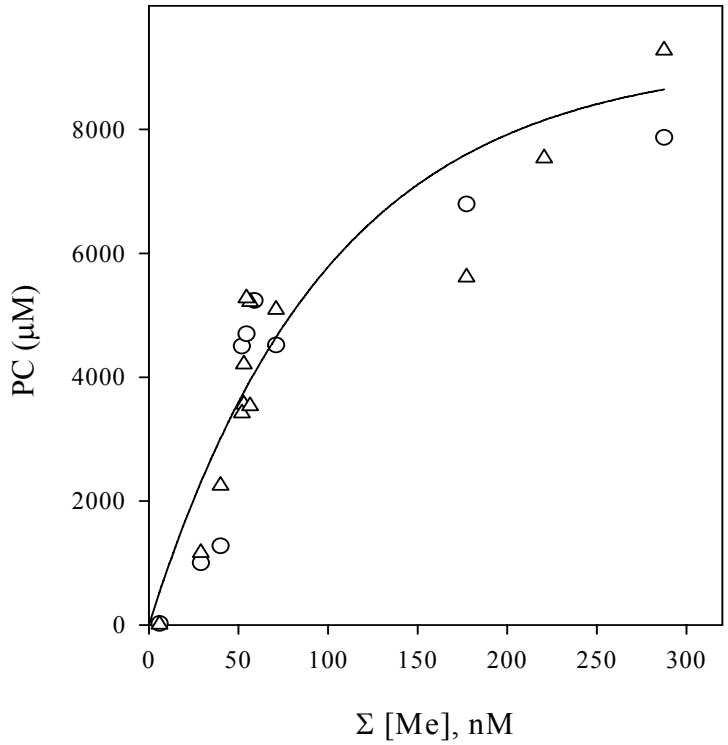

Fig. 4.7. Relationship between PC concentration in *T. weissflogii* cells and degree of metal contamination in the elutriates of the twelve sediment samples. ∑[Me] = [Cd] + [Cu] + [Pb]. Different symbols refer to two independent incubations in the same sample of elutriate. Initial cell density = 1 x 10^3 cells mL^{-1}; 6 day exposure.

Several papers report the use of analogous detoxifying peptides, metallothioneins, as biomarker of metal exposure in marine organisms at different trophic levels, including oysters (Geffard et al, 2003; Geffard et al, 2007), mussels (Donnini et al., 2007), crabs and clams (Martin-Diaz et al., 2007a; 2007b) and fishes (Jimenez-Tenorio et al, 2007). All of these show a significant relationship between metallothionein induction and metal concentration in the sediments. As suggested by Geffard et al., (2007) in a study carried out on larvae of *Crassostrea gigas* exposed to elutriates of metal-contaminated marine sediments, metallothioneins constitute a more sensitive biological indicator of metal stress than other physiological endpoints, behaving as an early biomarker of metal exposure. Analogously, the

present finding that PC induction in three species of marine diatoms occurs before than the growth rate can be affected suggests that also PC behave as an early warning response.

It must be underlined that PC concentration in autotrophic protists provides a gross index of the overall metal stress, rather than the response to a particular metal ion.

Results also show a different ability among the species to produce PC when exposed to sediment elutriates, depending on exposure conditions. Two of species assayed (*T. weissflogii* and *S. costatum)* exhibited a higher PC response during the 6-day exposure in the exponential growth phase compared to the 5-hour exposure in a not-growing condition, while for *P. tricornutum* the opposite was true. Hence, in designing suitable bioassays based on PC synthesis, it is important to choose the exposure conditions in dependence on a particular phytoplanktonic species.

Taken together, results show that *T. weissflogii* in the exponential growth phase was a more sensitive organism to be used in bioassays based on PC. In agreement, the high PC production in this diatom in response to metal exposure has been reported by other authors, (Ahner et al., 1995; Ahner et al., 2002), which hypothesized the occurrence in this diatom of a higher activity of the enzyme phytochelatin synthase. The application of the bioassay carried out with *T. weissflogii* shows that PC production in this diatom is consistent with the degree of metal contamination in the elutriates of sediments collected in a polluted coastal area, thus validating in the field the feasibility of using PC as a biomarker of metal pollution. It must be underlined that PC concentration in autotrophic microorganisms provides a gross index of the overall metal stress, rather than the response to a particular metal ion. In fact, combination of metals, acting with antagonistic and/or synergistic effects on PC induction (Wei et al, 2003; Kawakami et al., 2006b), is likely to occur in these complex matrices. Metal bioavailability also depends on the metal speciation, which is dominated by the presence of organic ligands. Mucha et al. (2003) in a study comparing the response of three species of microalgae to the

elutriate exposure, suggested that organic ligands released by sediments can affect metal bioavailability either through the metal sequestration which would reduce toxicity and/or an enhanced metal bioavailability due to the uptake of metal-ligand complexes. Thus, bioassays are necessary to measure the impact of heavy metals released by suspended marine sediments on living organisms. In the present study, the use of PC in marine diatoms as a biomarker of metal exposure is proposed, providing a tool to asses the potential toxic effects of the re-suspension of contaminated sediments. Such approach could be a complementary tool to classical bioassays based on the growth response in phytoplankton, and could supply interesting items in ecotoxicology, as PC constitute an early warning response, specific for metal stress.

4.4. Evaluation of phytochelatins synthesis and Dissolved Gaseous Mercury (DGM) production in *T. weissflogii* exposed to mercury

4.4.1. Effect of mercury exposure on growth rate of T. weissflogii

The effect of mercury on growth rate of *T. weissflogii* was investigated by growing cells in culture media at increasing Hg concentration (initial cell density $1x10^3$ cells mL^{-1}). The growth rate of the control culture (no Hg added) was about 1.0 ± 0.1 doublings day^{-1} (n=3). In the range of Hg from 5 to 500 nM the growth rate gradually decreased, reflecting the inhibition of cell growth under mercury exposure (Fig. 4.8).

Exponential growth was observed in all the cultures during 6 day exposure, but the Hg addition lengthened the lag phase. It was extrapolated that the 50% inhibition of growth rate occurred at an initial [Hg] = 250 nM, whereas inhibitions lower than 20% occurred for [Hg] $\leq$ 150 nM. In order to avoid toxic effects during mercury exposures, well tolerated Hg dosages ere used, never exceeding the dose of 150 nM in the exposure experiments.

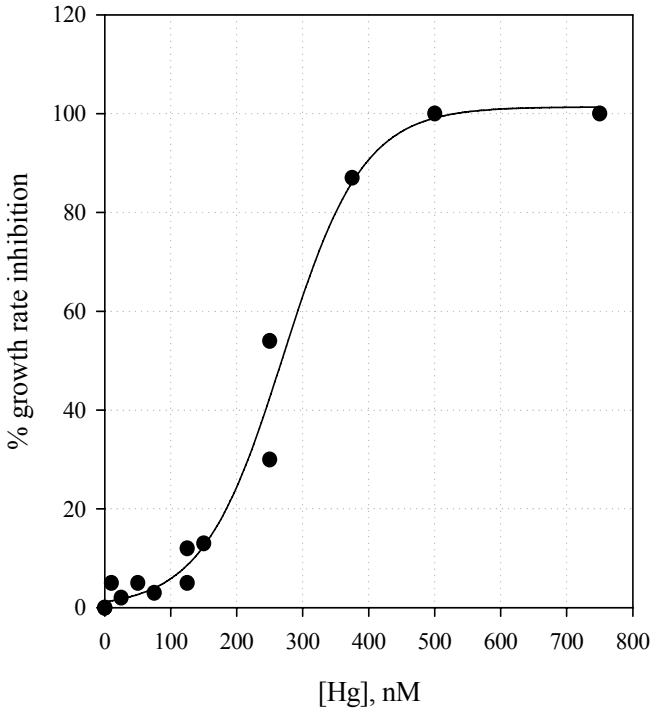

Fig. 4.8. Percentage of inhibition of the growth rate of *T. weissflogii* as a function of the mercury added to the medium.

4.4.2. Two-day exposure of T. weissflogii to mercury

The pattern of the non protein thiols pool in response to mercury exposure was studied by monitoring the concentration of glutathione, γ-Glu-Cys and PC in cells of *Thalassiosira weissflogii* exposed for two days to increasing Hg concentrations, from 5 to 150 nM. The cells responded to the Hg exposure by increasing the total level of the non protein thiols pool: besides glutathione and γ-Glu-Cys peptides, which are constitutively expressed in the cell, HPLC analysis showed the occurrence of PC. As shown in Fig. 4.9, glutathione was the main thiol in the entire range of Hg concentration and its intracellular concentration increased rapidly already at low Hg concentration. At 150 nM Hg, the amount of glutathione was two fold with respect to that found in the non treated cells.

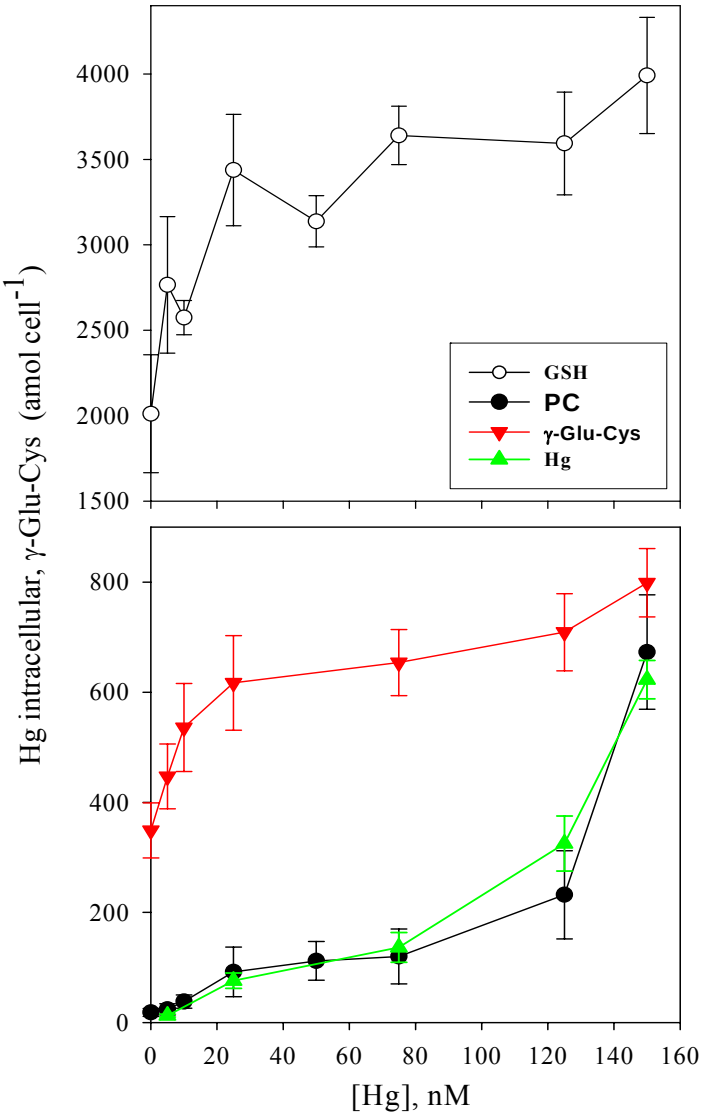

Fig. 4.9. Behaviour of non protein thiols in cells of *T. weissflogii* exposed to increasing mercury concentrations.

The concentration of the γ-Glu-Cys peptide was significantly less than that of glutathione both in the control (no Hg added) and in Hg treated cells, but its level increased with increasing Hg concentration in solution. Hg exposure also induced the synthesis of PC but, under these experimental conditions, they were detectable at [Hg] ≥ 25 nM. PC cellular pool increased by following a dose-response relationship till to reach the value of 673 ± 10^4 amol $cell^{-1}$ at 150

nM Hg. The cellular pool of peptides was composed mainly by PC_2 (85-100%), the remaining amount being polymerized as PC_3 (0-15%). The predominance of the pentapeptide and the inability to synthesize oligomers with $n > 3$ was observed in all cultures, irrespectively of the Hg dose.

Although it is well known that marine phytoplankton can synthesize PC in response to a variety of metal ions (Ahner et al., 2002; Kawakami 2006a), systematic studies regarding their capability to synthesize PC in response to mercury are lacking. Howe and Merchant (1992), in a study examining the ability of the green microalga *Chlamydomonas reinhardtii* to produce metal-binding peptides in response to Cd, Hg or Ag, reported that Hg-treated cells exhibited a transient but striking increase in the glutathione levels, however they were not able to accumulate measurable amount of PC. Recently, much more information has become available on the effects of Hg on the non-protein thiols pool in plants (Gupta et al., 1998; Iglesia-Turino, 2006; Israr, 2006; Rellan-Alvarez, 2006). Among these papers, a general agreement on the involvement of glutathione in Hg detoxification can be registered. Only a paper (Gupta et al., 1998) reports that, besides glutathione, PC can play a role in the Hg cellular sequestration in two species of aquatic plants.

In the present study, assays of intracellular Hg showed that the metal concentration increased with the Hg exposure and the concentration of PC was similar to that of intracellular Hg, exhibiting a molar ratio PC-SH : Hg close to 1. Since *in vitro* studies have shown that PC binds Hg with a steichiometry of two SH groups for one metal ion (Mehra et al., 1996), it seems that the amount of PC synthesized in this microorganism during a 2-day exposure is not sufficient to sequester intracellular mercury ions. The finding that cellular glutathione increased in response to Hg exposure, can account for a role in the intracellular mercury sequestration, in addition to PC.

4.4.3. Time course of non protein thiols pool and mercury accumulation

Exponentially growing cultures of T. weissflogii exposed to 150 nM Hg were assayed at time intervals for their intracellular concentration of Hg and non protein thiols (Fig. 4.10).

The time course of the $[Hg]_{intr}$ showed a rapid uptake of the metal, occurring during the first day of exposure, thereafter there was no further increase at longer exposures. The PC cellular concentration, after reaching a maximum value at the first day, decreased with exposure time, until to be halved at the 7th day of exposure. This finding indicates that PC synthesis occurs quickly, as soon as the metal is uptaken by the cells, thereafter, the lowering of its concentration suggests the occurrence of a process of degradation and/or export, as reported by other authors (Lee et al., 1996) for Cd-PC complexes induced in the same diatom.

Glutathione assays showed a transient increase of its intracellular concentration in the Hg-treated cells compared to that measured in the control culture, occurring during the first 2 days of exposure. An increase of 65% and 137% was calculated at the 1st and at the 2nd day, respectively. At the end of the experiment, the glutathione level in the Hg-treated cells was restored to values similar to those of the untreated cells. A pattern similar to that of GSH was observed for the γGlu-Cys peptides, which exhibited an increase in the Hg-treated cells compared to the untreated ones, of the 145% and 103% at the 1st and at the 2nd day, respectively.

The restoring of glutathione to basal levels could imply the occurrence of a process of release of this thiol. Accordingly, Tang et al. (2005) demonstrated an extracellular release of glutathione by *T. weissflogii* cells under copper stress.

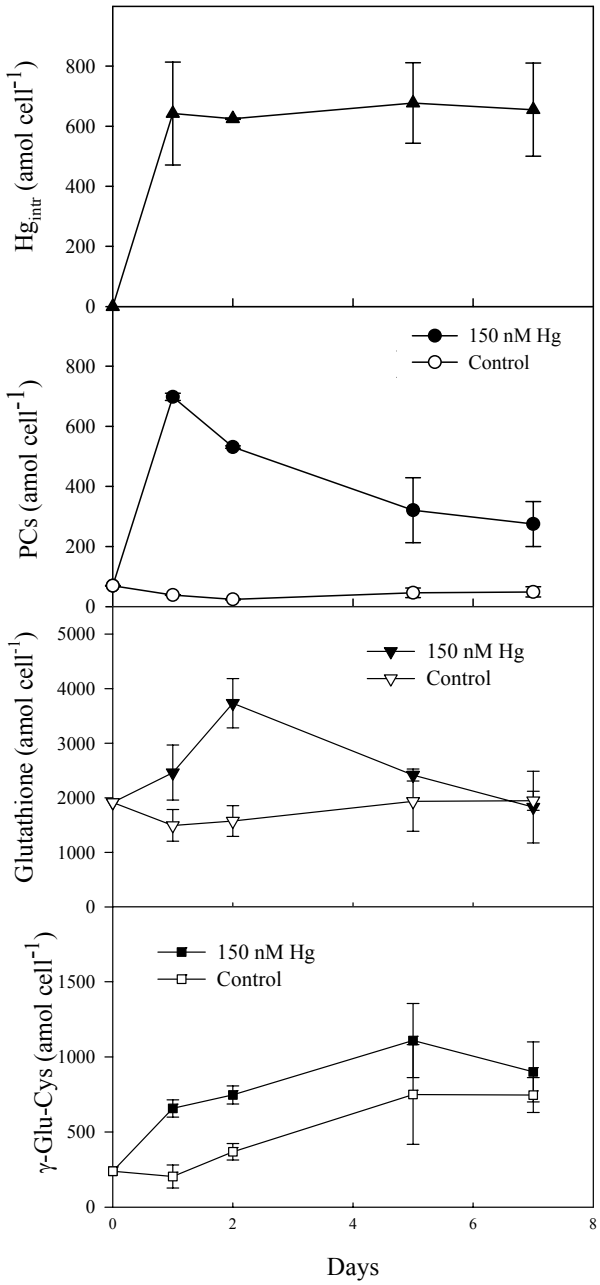

Fig. 4.10. Time course of non protein thiols pool and mercury accumulation in *T. weissflogii* exposed to 150 nM Hg.

The present results seems to suggest a mechanism in which the Hg taken up by cells at the beginning of the exposure can form Hg-PC complexes; these complexes could transfer the metal ion into the newly synthesized PC forming more stable Hg-PC complexes, which could,

in turn, be released and/or be degraded more slowly. The occurring of a similar mechanism for Hg sequestration is supported by an *in vitro* study demonstrating that GSH can transfer Hg into PC at increasing longer chain length (Mehra, 1996).

The initial formation of metal-glutathione complexes followed by a transfer to the metal induced PC has been also hypothesized to occur in *P. tricornutum* under Cd or Cu exposure (Morelli et al., 2002; Morelli and Scarano, 2004). In the present research, the fact that $[Hg]_{intr}$ remained almost constant during the entire incubation time while the PC concentration lowered, can suggest the presence of a process of transfer of part of the intracellular Hg initially sequestered by PC, or possibly by glutathione and γ-Glu-Cys peptides, to other, more stable, intracellular ligands. This hypothesis is supported by recent findings reported by Kelly et al. (2007), that have demonstrated the capability of a number of eukaryotic microalgae to biotransform Hg^{2+} into β-HgS at varying degrees and to accumulate this metal species in the cell.

In conclusion, the time course of the non protein thiols pool and $[Hg]_{intr}$ shows that PC, glutathione and γ-Glu-Cys peptides represent a rapid cellular response to mercury exposure; however, their role in Hg detoxification seems to lose importance at longer incubation times.

4.4.4. DGM production in T. weissflogii exposed to 5 nM mercury

At low mercury concentration, at which the PC synthesis doesn't seems to be involved, the intracellular Hg concentration followed anyhow a decreasing trend, starting from the beginning of exposure, towards longer incubation times. Thus, at 5 nM Hg_{add}, the $[Hg]_{intr}$ decreased from 20.6 ± 2.8 amol $cell^{-1}$ at the 1st day, to 6.6 ± 1.1 amol $cell^{-1}$ at the 7th day of exposure. This trend can be due, at least in part, to dilution by cell duplication, nevertheless the occurrence of a process of loss of Hg cannot be excluded. In the literature it has been reported that aquatic microorganisms, mainly bacteria but also eukaryotic phytoplankton

(Ben-Bassat and Mayer, 1977, 1978; Mason et al., 1995) are capable to transform ionic Hg to volatile forms. The existence of a similar process of Hg transformation could concur, to the observed lowering of the $[Hg]_{intr}$.

In order to investigate whether the marine diatom *T. weissflogii* is able to produce dissolved gaseous mercury (DGM), the mercury distribution in a culture of this diatom inoculated in a medium previously spiked with 5 nM Hg, was measured during the exponential growth phase. The pattern of $[Hg]_{diss}$ in the presence and in the absence of *T. weissflogii*, together with that of the Hg taken up by cells (Hg_{cell}), is reported in Fig. 4.11.

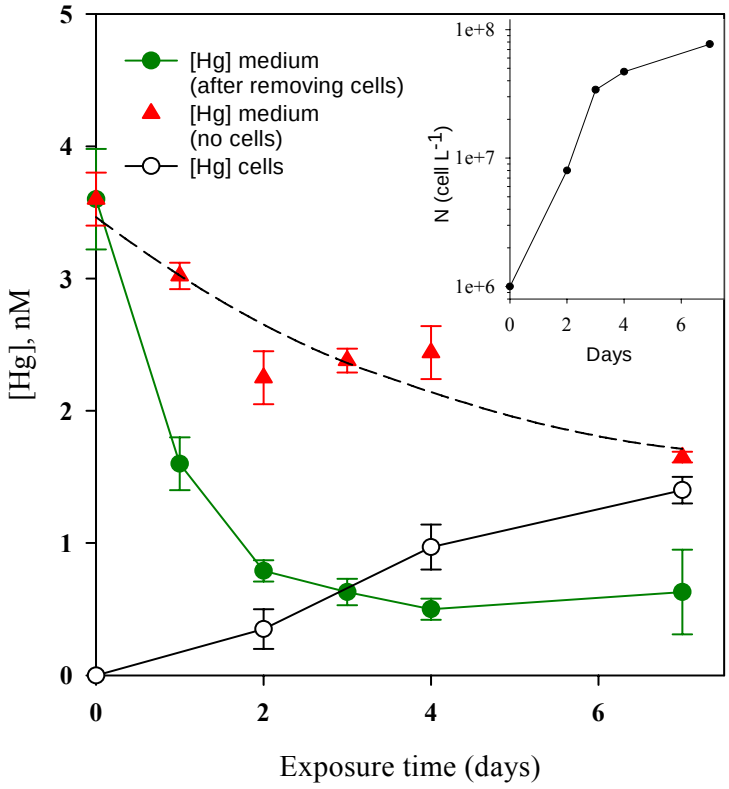

Fig. 4.11. Pattern of mercury concentration in cells of *T. weissflogii*, in the culture medium after removing cells and in the medium without cells.

The figure shows that the cell addition dramatically lowered the $[Hg]_{diss}$ in solution, concomitantly with an increase in cellular density (see insert of Fig. 4.11) and the fraction of

Hg associated to cells (Hg_{cell}) increased with incubation time, as expected due to the cell growth. However, it can be calculated that, during the exponential growth phase, this Hg amount is not sufficient to explain the loss of $[Hg]_{diss}$ in solution.

The contribution of the mercury volatilization to this loss of $[Hg]_{diss}$ in solution was investigated measuring the DGM production both in the culture *in toto* and in the culture medium after removal of the cells by filtration in order to discriminate between the biotic contribution (due to the cells of *T. weissflogii*) to the mercury volatilization and that of the cellular exudates in the medium. Cellular exudates are well known for their photo-sensitising role in marine photochemical reactions (Costa and Liss, 1999; Lanzillotta et al., 2004). Samples were analyzed under both dark and light conditions to compare the efficiency of the two DGM production processes.

DGM under light conditions was measured exposing samples at the same light intensity (100 μmol photons × m^{-2} × s^{-1}) used for the growth of *T. weissflogii*. This light intensity is particularly low with respect to that of the solar radiation, which can reach 400 W m^{-2}. These exposure conditions allow to maintain at low values the DGM production resulting from abiotic reactions of photoproduction involving dissolved organic matter comprising cellular exudates.

Results suggested a significant participation of the cells of *T. weissflogii* in mercury volatilization. The values of DGM production recorded at day 4th of exposure are reported in Fig. 4.12. A meaningful DGM production occurred both under dark and light conditions.

The culture *in toto* presented a DGM production in the dark of the order of 7.0 pmol h^{-1} L^{-1}, that reached values of 11.7 pmol h^{-1} L^{-1} under light conditions. The DGM production both in the dark and in lightness decreased in the culture medium after removal of the cells by filtration, showing values respectively of 3.0 pmol h^{-1} L^{-1} and 6.1 pmol h^{-1} L^{-1}, highlighting the role of the living cells in mercury reduction processes.

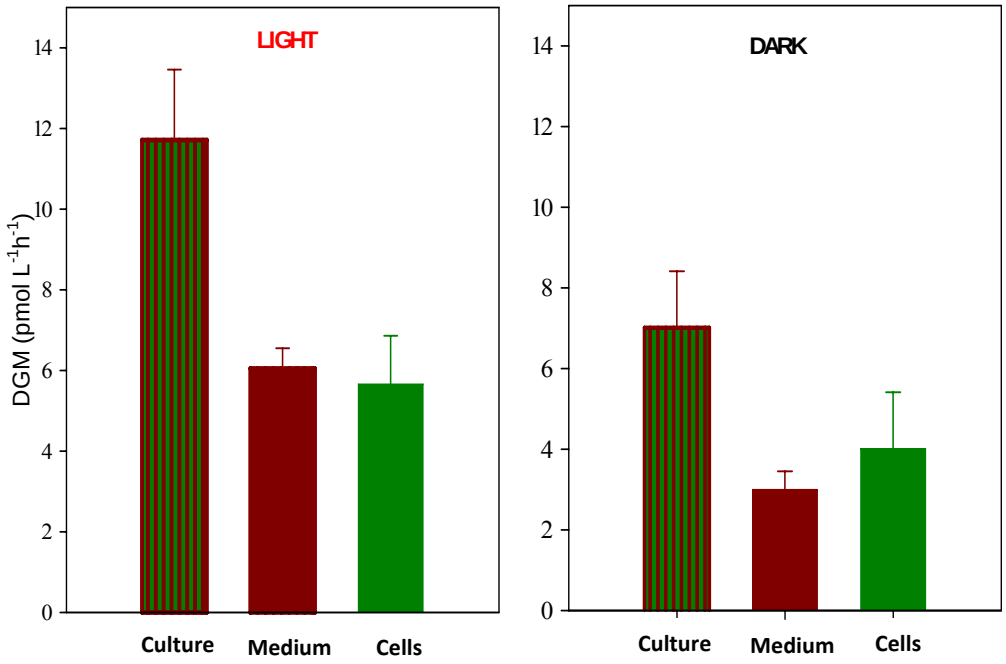

Fig. 4.12. DGM production in light and dark conditions recorded at day 4th of Hg exposure of the culture (cells + culture medium), of the culture medium and of the cells (culture – culture medium) of *T. weissflogii.*

To further confirmate these results the correlation between the percentage of the $[Hg]_{diss}$ in the medium that was volatilized by the cells during 1 hour (%DGM) and the cellular density of *T. weissflogii* was tested; moreover the percentage of the $[Hg]_{diss}$ in the medium that was volatilized by the cells during 1 hour was also drawn as a function of the dissolved mercury concentration in the medium (Fig. 4.13). Figure shows that there is a significant positive correlation between the cellular density and the %DGM, both in light conditions ($p < 0.01$) and in the dark ($p < 0.001$).

On the contrary no correlation exists between the %DGM and the dissolved mercury concentration in the medium. These results are interesting because strengthen the role of *T. weissflogii* in the mercury reduction process.

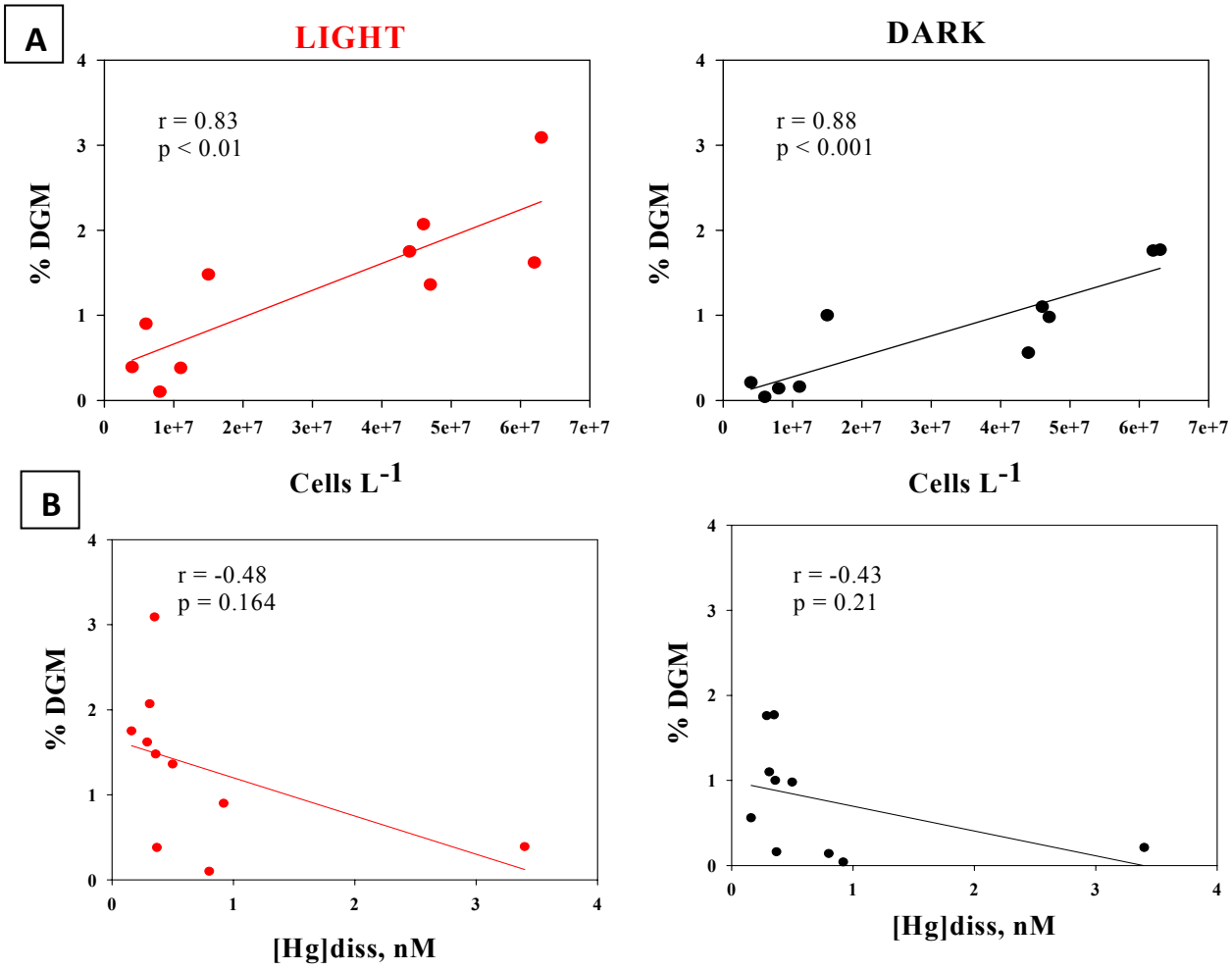

Fig. 4.13. **A.** Correlation between the percentage of the $[Hg]_{diss}$ in the medium volatilized in light and dark conditions by the cells of *T. weissflogii* during 1 hour (%DGM) and the cellular density of *T. weissflogii*; **B.** Correlation between the percentage of the $[Hg]_{diss}$ in the medium volatilized in light and dark conditions by the cells of *T. weissflogii* during 1 hour (%DGM) and the dissolved mercury concentration in the medium.

To further confirm this issue the DGM production was measured in cultures of *T. weissflogii* (exposed to the same mercury concentration) both alive and killed with formaldehyde in order to eliminate the cell contribution to the mercury reduction process. Results are reported in Tab. 4.5 together with the DGM production of the culture medium after removing cells by filtration.

	DGM pmol h^{-1} L^{-1}	
	Light	**Dark**
Culture with cells alive (1)	**8.7 ± 0.8**	**4.7 ± 1.8**
Culture with cells killed (2)	**4.6 ± 0.7**	**0.3 ± 0.2**
Culture medium (3)	**4.1 ± 0.9**	**0.2 ± 0.1**
Cells alive (1)-(3)	**4.6 ± 0.8**	**4.5 ± 0.9**
Cells killed (2)-(3)	**0.5 ± 0.7**	**0.1 ± 0.2**

Tab. 4.5. DGM produced by cultures of *T. weissflogii* (exposed to 5 nM Hg) both alive and killed with formaldehyde and DGM production of the culture medium after removing cells by filtration.

This experiment produced information which corroborate the previous findings. The DGM production of the culture of *T. weissflogii* with cells alive was significantly higher than that measured in the culture medium alone, both in light and dark conditions. On the contrary, the DGM production of the culture with formaldehyde-killed cells exhibited values of DGM similar to those obtained after cells removal. These results clearly demonstrate the significant contribution of living cells in mercury volatilization.

It is important to note that in our experimental conditions the contribute of the living cells to the DGM production is independent from the light, being 4.6 ± 0.8 pmol h^{-1} L^{-1} in the light and 4.5 ± 0.9 pmol h^{-1} L^{-1} in dark conditions. These findings are consistent with those of Devars et al. (2000) who found that the ability to volatilize mercury by the freshwater microalga *Euglena gracilis* was independent from light.

Our results suggesting that *T. weissflogii* is capable of reducing Hg^{2+} to Hg^0 are in agreement with previous studies reporting that eukaryotic microorganisms can reduce mercury (Ben-

Bassat and Mayer, 1977, 1978; Bentz 1977). Amyot et al. (1994) and Vandal et al. (1991, 1993) found a correlation between chlorophyll *a* concentration and Hg^0 formation rate suggesting that there is a link between productivity and mercury reduction. Mason et al. (1995) carried out experiments with both laboratory monocultures of a number of phytoplanktonic species, including *T. weissflogii*, and demonstrated their capability of reducing Hg^{2+} to Hg^0, although the rate of reduction was insufficient to account for the observed reduction rates in incubated field samples; the rate of DGM production measured by these authors for *T. weissflogii* (0.29 amol $cell^{-1}$ d^{-1}) was comparable to that measured in the present research (2.6 amol $cell^{-1}$ d^{-1}) at similar cellular densities (5-7 x 10^7 cells mL^{-1}), taking into account the ten fold higher $[Hg]_{add}$, used in the present study.

Very little was found in the literature on the mechanism involved in the mercury reduction in eukaryotic microorganisms. Hg^0 production could involve cell surface reduction, similar to that found for other trace metals (Jones et al., 1986), rather than a gene encoded Hg resistance mechanism, as in the case of prokaryotic microorganisms (Barkey et al., 2003). Ben-Bassat and Mayer (1977) isolated from crude extracts of the green alga *C. Pyrenoidosa* an intracellular fraction (molecular weight < 1200 Da) responsible of Hg reduction, but its nature remains unknown.

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CHAPTER 5

CONCLUSIONS

The main results of this research on the most important mechanisms of detoxification acting in autotrophic protists to cope with heavy metal stress can be summarized as follows:

- the response of the marine diatom *P. tricornutum* to metal exposure suggested that the production of intracellular peptides capable to bind metal ions, named phytochelatins (PC), represent a useful biomarker for the assessment of metal bioavailability in marine waters.
- Experiments carried out to compare the ability of five taxa of autotrophic protists (*P. tricornutum*, *T. weissflogii, S. costatum*, *D. tertiolecta*, *E. huxleyi*) to produce PC when exposed to heavy metals showed that the PC response is specie-specific. Diatoms were shown to be more responsive to metal stress than the other microorganisms tested.
- Studies on metal contamination in sediments showed a good relationship between PC production and elutriate concentration in *P. tricornutum, T. weissflogii* and *S. costatum* cells, both in short-term and in long-term incubations, demonstrating that PC represent an appropriate biomarker of metal exposure, which can be utilized also in a complex matrix such as the elutriates of marine sediments. Moreover results showed that *T. weissflogii* in the exponential growth phase was the more sensitive organism to be used in bioassays based on PC.
- Finally, experiments of mercury exposure carried out using *T. weissflogii* showed that this diatom is able to activate a process of mercury reduction, producing measurable amounts of dissolved gaseous mercury when exposed even at low mercury concentration ($[Hg]_{add}$ = 5 nM). At increasing mercury concentrations ($[Hg]_{add}$ = 10-

150 nM), the process of volatilization of the metal appeared to be insufficient to prevent mercury intracellular accumulation. In this case, the mercury accumulated by the cells would induce a general increase in the actual pool of glutathione and γ-Glu-Cys, besides to induce an ex-novo synthesis of PC.

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